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(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF MICHIGAN [US/US]; Room 2071, 3003 South State Street, Ann Arbor, MI 48109–1280 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NABEL, Gary, J. [US/US]; 385 Meadow Creek Drive, Ann Arbor, MI 48105 (US). CHEN, Jian–Jun [CN/US]; 1156 Mcintyre Drive, Ann Arbor, MI 48105 (US).

(74) Agents: KONSKI, Antoinette, F. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304–1018 (US).

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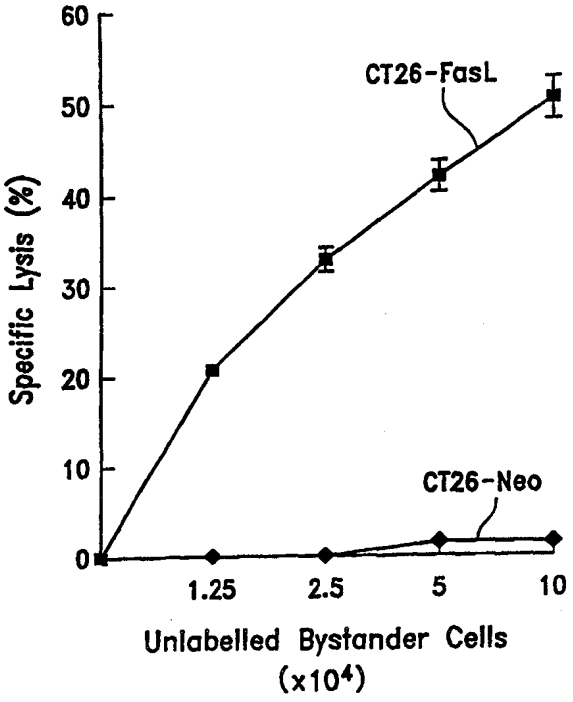
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING THE PROINFLAMMATORY RESPONSE

(57) Abstract

This invention provides a method for inhibiting a proinflammatory response in a suitable cell mixture, by administering to the mixture an effective amount of an immunosuppressive agent which acts to inhibit the proinflammatory activity of FasL. In some embodiments, an effective amount of FasL is coadministered with the immunosuppressive agent. The method can be practiced *in vitro, ex vivo* or *in vivo*. This invention also provides a method of inhibiting a FasL–mediated proinflammatory response in a subject, comprising administering to the subject an effective amount of an immunosuppressive agent that specifically inhibits the immunostimulatory effect of FasL. The immunosuppressive agent can be any agent which inhibits the immunostimulatory function of Fas. Such agents include, but are not limited to anti–sense molecules that inhibit endogenous FasL expression, soluble Fas receptors or variant thereof, ribozymes that inhibit the endogenous expression of FasL, drugs that inhibit FasL signalling, agents that induce the endogenous expression of TGF–β, such as cyclosporin or thrombospondin 1, polynucleotides coding for an immunosuppressive agent such as TGF–β, or TGF–β protein or polypeptide. This invention further provides a method for identifying agents which modulate FasL stimulation of a proinflammatory response.



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METHODS AND COMPOSITIONS FOR INHIBITING THE PROINFLAMMATORY RESPONSE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of under 35 U.S.C. § 119(e) of United States provisional application number 60/052,829, filed July 17, 1997, the contents of which are hereby incorporated by reference into the present disclosure.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was supported in part by a grant from the National Institutes of Health (NIH R01 AI31946; D.K.B.). Accordingly, the U.S. government may have rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application, publications are referred to by bibliographic citation, patent number or publication number. The disclosures of these publications are hereby incorporated by reference in their entirety into the present disclosure to more fully describe the state of the art to which this invention pertains.

The CD95 or Fas antigen is a cell surface receptor which transduces apoptotic signals into cells. Itoh, N. et al. (1991) *Cell* **66**:233. The physiological ligand of Fas, Fas-ligand (FasL), is a type 2 membrane protein which can transduce this signal upon cell contact (Suda, T. et al. (1993) *Cell* **75**:1169) or in a soluble form. Nagata, S. and Goldstein, P. (1995) *Science* **267**:1449. The primary function of the Fas-FasL system is thought to be the maintenance of homeostasis in the immune system by the clonal deletion of autoreactive lymphocytes in peripheral lymphoid tissues and the elimination of expanded lymphocyte populations. Nagata, S. and Goldstein, P. (1995) *Science* **267**:1449; Brunner, T. et al. (1995) *Nature* **373**:441; Dhein, J. et al. (1995) *Nature* **373**:438; and Ju, S. et al. (1995) *Nature* **373**:444. FasL expression in normal tissue is restricted to T lymphocytes, macrophages, the cornea, the iris, ciliary bodies, the retina and Sertoli cells

(Wong-Staal, F. (1994) *HIV- Advances in Research and Therapy* **4**:3; and Griffith, T. et al. (1995) *Science* **270**:1189). Through its ability to suppress both cellular and humoral immunity (Brunner, T. et al. (1995) *Nature* **373**:441; Dhein, J. et al. (1995) *Nature* **373**:438; Ju, S. et al. (1995) *Nature* **373**:444; and Arai, H. et al. (1997) *Nat. Med.* **3**:843), FasL has been implicated in maintenance of the immune-privileged status in the eye (Griffith, T. et al. (1995) *Science* **270**:1189) and testis (Bellgrau, et al. (1995) *Nature* **377**:630). It has also been suggested that expression of FasL confers immune suppression in malignancy as a mechanism to evade immune detection (Strand, S. et al. (1996) *Nat. Med.* **2**:1361; O'Connell, J. et al. (1996) *J. Exp. Med* **184**:1075; and Hahne, M. et al. (1996) *Science* **274**:1363). FasL has therefore been proposed as a gene product which may be useful in the setting of cell or organ transplantation, as evidenced by its ability to delay the rejection of allogeneic cells (Lau, M. et al. (1996) *Science* **273**:109). However, the ability of FasL to prolong foreign tissue engraftment is complicated and contraindicated by the proinflammatory effects of FasL (Arai, H. et al. (1997) *Nat. Med.* **3**:843). Syngeneic tumor cells which express FasL are also rapidly rejected by inflammatory cells *in vivo* (Arai, H. et al. (1997) *Proc. Natl. Acad. Sci USA* **94**:13862; and Seino, K-I. et al. (1997) *Nat. Med.* **3**:165). Furthermore, expression of FasL on myotubes or pancreatic islets of transgenic mice induced a potent granulocytic infiltration which unexpectedly accelerated graft rejection (Kang, et al. (1997) *Nat. Med.* **3**:738; Allison, J. et al. (1997) *Proc. Natl. Acad. Sci. USA* **94**:3943; and Kang, S.M. et al. (1996) *Science* **278**:1322). It has been postulated that differences in the effect of FasL at distinct anatomic sites may be caused by secondary factors which modulate its function (Green, D.R. et al. (1997) *Proc. Natl. Acad. Sci. USA* **94**:5986). Thus, a need exists to identify factors which could explain the paradoxical effects of FasL in immune-privileged sites and immunocompetent tissues. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

It is an aspect of this invention to provide a method for inhibiting a proinflammatory response in a suitable cell mixture, by administering to the mixture an effective amount of an immunosuppressive agent which acts to inhibit the proinflammatory activity of FasL. In some embodiments, an effective amount of FasL is coadministered

with the immunosuppressive agent. In a further embodiment, the cell mixture comprises neutrophil cells. The method can be practiced *in vitro*, *ex vivo* or *in vivo*.

It is a further aspect of this invention to provide a method of inhibiting a FasL-mediated proinflammatory response in a subject, comprising administering to the subject an effective amount of an immunosuppressive agent that specifically inhibits the immunostimulatory effect of FasL. The immunosuppressive agent can be any agent which inhibits the immunostimulatory function of FasL. Such agents include, but are not limited to, anti-sense molecules that inhibit endogenous FasL expression, soluble Fas receptors or variants thereof, ribozymes that inhibit the endogeneous expression of FasL, drugs that inhibit FasL signalling, agents that induce the endogenous expression of TGF-β, such as cyclosporin or thrombospondin 1, polynucleotides coding for an immunosuppressive agent such as TGF-β, or TGF-β protein or polypeptide.

It is to be understood, although not always explicitly stated that the above agents, molecules and compositions can be administered alone or in combination with a carrier, e.g., a pharmaceutically acceptable carrier.

It is a further aspect of this invention to provide a method for identifying agents which modulate FasL stimulation of a proinflammatory response, by:

(a) contacting a target cell mixture and a control cell mixture with FasL and an immunosuppressive agent;

(b) contacting the target cell mixture with a candidate therapeutic agent;

(c) assaying the target cell mixture for localized proinflammatory response; and

(d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates localized FasL stimulation of the proinflammatory response.

In a still further aspect of this invention, provided is a method for identifying agents which modulate FasL stimulation of a proinflammatory response, by:

(a) contacting a target cell mixture and a control cell mixture with FasL;

(b) contacting the target cell mixture with a candidate therapeutic agent and the control cell mixture with an immunosuppressive agent;

(c) assaying the target cell mixture for localized proinflammatory response; and

(d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates localized FasL stimulation of the proinflammatory response, or alternatively,

(a) contacting a target cell mixture and a control cell mixture with an effective amount of immunosuppressive agent;

(b) contacting the target cell mixture with an effective amount of candidate therapeutic agent and the control cell mixture with an effective amount of FasL:

(c) assaying the target cell mixture for localized proinflammatory response; and

(d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates FasL-mediated stimulation of the proinflammatory response.

The above methods can be practiced *in vitro* and/or *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A to 1C shows neutrophil-mediated destruction of $CD95^+$ CT26 but not $FasL^-$, CT26 cells *in vitro*.

Figure 1A shows neutrophil dose-dependent killing of $FasL^+$ CT26 cells. Figure 1B shows neutrophil effector-mediated killing. Figure 1C shows cytolysis mediated by neutrophils from syngeneic animals.

Figures 2A and 2B show FasL interaction is required for neutrophil cytolytic function.

Figure 2A shows blockade of neutrophil cytotoxicity by Fas-Fc fusion protein, prepared as described below and in Brunner, T. (1995) *Nature* **373**:441. CT26-FasL target cells were labeled with ^{51}Cr and mixed with neutrophils at an effector-to-target ratio of 50:1. Increasing amounts of mouse Fas-Fc protein, control human Ig or anti-Fas3 antibody were added to the medium to neutralize the killing.

Figure 2B shows induction of bystander neutrophil cytotoxicity against CT26-neo by CT26-FasL cells. CT26-neo target cells were labeled with ^{51}Cr and mixed with neutrophils at an E/T ratio of 100:1 in the presence of the indicated numbers of unlabeled CT26-FasL cells. Equal numbers of unlabeled CT26-neo cells were used as a negative control.

Figures 3A to 3E show inhibitory effects of recombinant TGF-β on neutrophil-mediated cytotoxicity and its role in the aqueous humor.

Figure 3A shows inhibition of neutrophil cytolysis by TGF-β1. Neutrophils were incubated with CT26-FasL cells at an E/T ratio of 100:1. Increasing amounts of human TGF-β1 (R&D Systems), human IL-10 (Genzyme) and GM-CSF (Immunex) were added to the culture and chromium releases assays were performed as in Figure 2.

Figure 3B shows lack of inhibition of FasL-mediated apoptosis by TGF-β1 in Jurkat cells. CT26-FasL were incubated with ^{51}Cr-labeled Jurkat cells ($1x10^4$/well) at a ratio of 10:1. Human TGF-βl (20 ng/ml) was added to the medium, and Fas-Fc fusion protein was used as a positive control. Cytotoxicity was measured by ^{51}Cr release after 4 hours of co-incubation. The data represent mean ±- SD from three assays.

Figure 3C shows inhibition of neutrophil-mediated cytolysis of CT26-FasL cells by preincubation of neutropbils with TGF-β1. Neutrophils were preincubated with TGF-β1 (20 ng/ml) or GM-CSF (20 ng/ml) for the indicated period and washed with 10 ml of medium three times. The percentage of inhibition was calculated relative to neutrophils preincubated with medium, TGF-β1, or GM-CSF for the same period of time. The data represent mean ± SD from three assays.

Figure 3D shows inhibitory effects of aqueous humor on neutrophil-mediated lysis of CT26-FasL cells. The indicated percentages of bovine aqueous humor or heat inactivated bovine aqueous humor (volume/volume) were added to the assay described in Figure 2A at an E:T of 100:1.

Figure 3E shows reversal of inhibitory effects of aqueous humor by neutralizing soluble TGF-β receptor protein. Mouse neutrophils were incubated with radiolabeled CT-26-FasL at a ratio of 50:1 and 40 µl of bovine aqueous human were added to the assay. The indicated concentrations of soluble human TGF-β1 receptor (TGF-β SRII/Fc, purchased from R&D Systems, Minneapolis, MN) or control human immunoglobulin were added to the assay as shown in Figure 1C.

Neutrophils (10^6/ml) were incubated with medium or human soluble FasL (400 ng/ml, Upstate Biotechnology) in the presence or absence of TGF-β1 (20ng/ml). Cells were harvested 19 hours after incubation, and the number of surviving cells was calculated

using Trypan blue exclusion. Cell viability was: 83.6±2% (medium), 85.4±3.4% (TGF-β1); 78.5±3.1% (FasL); or 80.9±2.0% (TGF-β+FasL).

Figure 4A provides the polynucleotide and predicted amino acid sequence of rat FasL (SEQ ID NOS: 1 and 2, respectively). The numbers above and below each line refer to the nucleotide position and amino acid positions, respectively. The putative transmembrane domain is underlined and four potential N-linked glycosylation sites (N-X-S/T) are indicated by asterisks. Figure 4B (SEQ ID NOS: 3 and 4) provides the nucleotide and amino acid sequence of soluble Fas receptor as provided in WO 95/13701, which also describes methods for recombinantly producing and purifying the soluble Fas receptor.

Figure 5 provides the polynucleotide and amino acid sequence of human FasL (SEQ ID NOS: 5 and 6) as described in WO 95/18819. The human FasL protein comprises an N-terminal cytoplasmic domain (amino acids 1-80), a transmembrane region (amino acids 81-105), and an extracellular domain (amino acids 106-281). The extracellular domain contains the receptor binding region. Soluble FasL polypeptides comprise all or part of the extracellular domain of a FasL protein, but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. A heterologous signal peptide can be fused to the N-terminus such that the soluble FasL is secreted upon expression. Soluble FasL can also include part of the transmembrane region or part of the cytoplasmic domain or other sequences, provided that the soluble FasL is capable of being secreted. Methods of recombinantly producing FasL or isolating soluble FasL from the supernatant of cells are described in Tanaka, et al. (1995) *EMBO J.* **14(6)**:1129.

Figure 6 provides the amino acid sequences of several proteins having FasL activity for use in the methods of this invention as provided below (SEQ ID NOS: 7 to 10). (See Figure 2(b) of Nagata, S. et al. (1995) *Science* **267**:1449.) Shown is the nucleotide and amino acid sequence of mutant FasL [mFasL(gld)] and wild type FasL (mFasL). The arrow indicates the position of the mutation in FasL of gld mice. Amino acid sequences of the corresponding region of the other members of the TNF family (TNF, LT-α, CD40L, CD27L and CD30L) are also shown. The amino acids of favored substitutions in more than four members are boxed.

Figure 7 provides the polynucleotide sequence coding for TGF-β.

Figures 8A and 8B show abrogation of FasL neutrophil cytolysis by p38 MAPK pharmacologic inhibitors, and p38 MAPK activation by FasL is inhibited by TGF-β.

Figure 9A shows the effects of p38 MAPK inhibitors on FasL-stimulated neutrophil cytotoxicity. Neutrophils were incubated with CT26-FasL cells at an E/T ratio of 50:1. Increasing amounts of commercialy available p38 MAPK inhibitors, SB203580 (IC_{50}, 350 nm, SmithKline Beecham), SB202190 (IC_{50}, 600nm, SmithKline Beecham), or an ERK kinase inhibitor (negative control), PD988059 (IC_{50}, 2μm) were added to media. Chromium release assays were prepared as in Figure 2.

Figure 2B shows activation and modulation of p38 MAPK in neutrophils by FasL and inhibition by TGF-β. In the left panel, detection of phosphorylated p38 MAPK by Western blot analysis (Sambrook et al, *Supra*). Human neutrophils were pretreated with human TGF-β1 (20ng/ml, lanes 3 and 4) or medium (lanes 1 and 2) at 4°C for 1 hour. Subsequently, the neutrophils were cocultured with 400 ng/ml of human FasL (Upstate Biotechnology, lanes 2 and 3) or medium (lanes 1 and 4) for 10 minutes at 37°C. Western blotting was performed using antibodies specific for phosphorylated p38 MAPK (New England BioLabs, Inc.). In the right panel, enzymatic activity of p38 MAPK was determined by phosphorylation of ATF2 substrate. Neutrophils were treated as described above. Cellular p38 MAPK activity was determined by phosphorylation of ATF2 by immunoprecipitated p38 according to manufacturer's instructions (New England Biolabs, Inc.). To verify that the change in the level of phosphorylated p38 MAPK and kinase activity did not result from variable amounts of protein, the total amount of p38 MAPK was examined by Western blotting using p38 kinase antibody according to manufacturer's instructions (New England Biolabs, Inc.).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA. These methods are described in the following publications. See, *e.g.*, Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the

series METHODS IN ENZYMOLOGY (Academic Press, Inc.); "PCR: A PRACTICAL APPROACH" (M. MacPherson et al. IRL Press at Oxford University Press (1991)); PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)); ANTIBODIES, A LABORATORY MANUAL (Harlow and Lane, eds. (1988)); and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures.

The terms "proteins", "peptides" and "polypeptides" are used interchangeably and are intended to include molecules containing amino acids linearly coupled through peptide bonds. The amino acids can be in the L or D form so long as the biological activity of the polypeptide is maintained. For example, the protein can be altered so as to be secreted from the cell for recombinant production and purification. These also include proteins which are post-translationally modified by reactions that include glycosylation, acetylation and phosphorylation. Such polypeptides also include analogs, alleles and allelic variants which can contain amino acid derivatives or non-amino acid moieties that do not affect the biological or functional activity of the protein as compared to wild-type or naturally occurring protein. The term amino acid refers both to the naturally occurring amino acids and their derivatives, such as TyrMe and PheCl, as well as other moieties characterized by the presence of both an available carboxyl group and an amine group. Non-amino acid moieties which can be contained in such polypeptides include, for example, amino acid mimicking structures. Mimicking structures are those structures which exhibit substantially the same spatial arrangement of functional groups as amino acids but do not necessarily have both the α-amino and α-carboxyl groups characteristic of amino acids.

"Muteins" are proteins or polypeptides which have minor changes in amino acid sequence caused, for example, site-specific mutagenesis or other manipulations; by errors in transcription or translation; or which are prepared synthetically by rational design. As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the α-amino group of another amino acid.

As used herein, the term "hydrophobic" is intended to include those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are non-polar.

Hydrophobic amino acids include Phe, Val, Trp, Ile and Leu. As used herein, the term "positively charged amino acid" refers to those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are positively charged. Positively charged amino acids include, for example, Lys, Arg and His.

"Native" polypeptides, proteins, or nucleic acid molecules refer to those recovered from a source occurring in nature or "wild-type".

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

The term "polynucleotide" means single and double stranded DNA, cDNA, genome-derived DNA, and RNA, as well as the positive and negative strand of the nucleic acid that are complements of each other, including anti-sense RNA. A "nucleic acid molecule" is a term used interchangeably with "polynucleotide" and each refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. It also includes known types of modifications, for example labels which are known in the art (e.g., Sambrook, et al. (1989) *supra.*), methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl carbamate, etc.), those containing pendant moieties, such as for example, proteins (including , e.g., nuclease, toxins, antibodies, signal peptides, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with

modified linkages (e.g., alpha anomeric polynucleotide, etc.), as well as unmodified forms of the polynucleotide. The polynucleotide can be chemically or biochemically modified or contain non-natural or derivatized nucleotide bases. The nucleotides may be complementary to the mRNA encoding the polypeptides. These complementary nucleotides include, but are not limited to, nucleotides capable of forming triple helices and antisense nucleotides. Recombinant polynucleotides comprising sequences otherwise not naturally occurring are also provided by this invention, as are alterations of wild type polypeptide sequences, including but not limited to, those due to deletion, insertion, substitution of one or more nucleotides or by fusion to other polynucleotide sequences.

A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well-known to those skilled in the art, it can be transcribed and/or translated to produce a polypeptide or mature protein. Thus, the term polynucleotide shall include, in addition to coding sequences, processing sequences and other sequences that do not code for amino acids of the mature protein. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

The term "recombinant" polynucleotide or DNA refers to a polynucleotide that is made by the combination of two otherwise separated segments of sequence accomplished by the artificial manipulation of isolated segments of DNA by genetic engineering techniques or by chemical synthesis. In so doing one may join together DNA segments of desired functions to generate a desired combination of functions.

An "analog" of DNA, RNA or a polynucleotide, refers to a macromolecule resembling naturally occurring polynucleotides in form and/or function (particularly in the ability to engage in sequence-specific hydrogen bonding to base pairs on a complementary polynucleotide sequence) but which differs from DNA or RNA in, for example, the possession of an unusual or non-natural base or an altered backbone. See for example, Uhlmann et al. (1990) *Chemical Reviews* **90**:543.

A "gene" is a hereditary unit that, in the classical sense, occupies a specific position (locus) within the genome or chromosome; a unit that has one or more specific effects upon the phenotype of the organism; a unit that can mutate to various allelic forms; a unit that recombines with other such units. Three classes of genes are now recognized: (1) structural genes that are transcribed into mRNAs, which are then translated into

polypeptide chains, (2) structural genes that are transcribed into rRNA or tRNA molecules which are used directly, and (3) regulatory genes that are not transcribed, but serve as recognition sites for enzymes and other proteins involved in DNA replication and transcription.

A "probe" is any biochemical labeled with radioactive isotopes or tagged in other ways for ease in identification. A probe is used to identify or isolate a gene, a gene product, or a protein. Examples of probes include, but are not limited to, a radioactive mRNA hybridizing with a single strand of its DNA gene, a DNA or cDNA hybridizing with its complementary region in a chromosome, or a monoclonal antibody combining with a specific protein.

"Hybridization" refers to hybridization reactions can be performed under conditions of different "stringency". Conditions that increase the stringency of a hybridization reaction are widely known and published in the art: see, for example, Sambrook, et al. (1989) *supra* . Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25 ° C, 37 ° C, 50 ° C, and 68 ° C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours and washes of increasing duration, increasing frequency, or decreasing buffer concentrations.

Hybridization reactions can be performed under conditions of different "stringency". In general, a low stringency hybridization reaction is carried out at about 40 ^{0}C in 10 x SSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50 ^{0}C in 6 x SSC, and a high stringency hybridization reaction is generally performed at about 60 ^{0}C in 1 x SSC.

A "stable duplex" of polynucleotides, or a "stable complex" formed between any two or more components in a biochemical reaction, refers to a duplex or complex that is sufficiently long-lasting to persist between the formation of the duplex or complex, and its subsequent detection. The duplex or complex must be able to withstand whatever conditions exist or are introduced between the moment of formation and the moment of detection, these conditions being a function of the assay or reaction which is being performed. Intervening conditions which may optionally be present and which may dislodge a duplex or complex

include washing, heating, adding additional solutes or solvents to the reaction mixture (such as denaturants), and competing with additional reacting species. Stable duplexes or complexes may be irreversible or reversible, but must meet the other requirements of this definition. Thus, a transient complex may form in a reaction mixture, but it does not constitute a stable complex if it dissociates spontaneously or as a result of a newly imposed condition or manipulation introduced before detection.

When stable duplexes form in an antiparallel configuration between two single-stranded polynucleotides, particularly under conditions of high stringency, the strands are essentially "complementary". A double-stranded polynucleotide can be "complementary" to another polynucleotide, if a stable duplex can form between one of the strands of the first polynucleotide and the second. A complementary sequence predicted from the sequence of a single stranded polynucleotide is the optimum sequence of standard nucleotides expected to form hydrogen bonding with the single-stranded polynucleotide according to generally accepted base-pairing rules.

A "sense" strand and an "antisense" strand when used in the same context refer to single-stranded polynucleotides which are complementary to each other. They may be opposing strands of a double-stranded polynucleotide, or one strand may be predicted from the other according to generally accepted base-pairing rules. Unless otherwise specified or implied, the assignment of one or the other strand as "sense" or "antisense" is arbitrary.

A linear sequence of nucleotides is "identical" to another linear sequence, if the order of nucleotides in each sequence is the same, and occurs without substitution, deletion, or material substitution. It is understood that purine and pyrimidine nitrogenous bases with similar structures can be functionally equivalent in terms of Watson-Crick base-pairing; and the inter-substitution of like nitrogenous bases, particularly uracil and thymine, or the modification of nitrogenous bases, such as by methylation, does not constitute a material substitution. An RNA and a DNA polynucleotide have identical sequences when the sequence for the RNA reflects the order of nitrogenous bases in the polyribonucleotide, the sequence for the DNA reflects the order of nitrogenous bases in the polydeoxyribonucleotide, and the two sequences satisfy the other requirements of this definition. Where at least one of the sequences is a degenerate oligonucleotide comprising an ambiguous residue, the two sequences are identical if at least one of the alternative forms of the degenerate

oligonucleotide is identical to the sequence with which it is being compared. For example, AYAAA is identical to ATAAA, if AYAAA is a mixture of ATAAA and ACAAA.

When comparison is made between polynucleotides, it is implicitly understood that complementary strands are easily generated, and the sense or antisense strand is selected or predicted that maximizes the degree of identity between the polynucleotides being compared. For example, where one or both of the polynucleotides being compared is double-stranded, the sequences are identical if one strand of the first polynucleotide is identical with one strand of the second polynucleotide. Similarly, when a polynucleotide probe is described as identical to its target, it is understood that it is the complementary strand of the target that participates in the hybridization reaction between the probe and the target.

A linear sequence of nucleotides is "essentially identical" or the "equivalent" to another linear sequence, if both sequences are capable of hybridizing to form duplexes with the same complementary polynucleotide. It should be understood, although not always explicitly stated that when Applicants refer to a specific polynucleotide, its equivalents are also intended. Sequences that hybridize under conditions of high stringency are more preferred. It is understood that hybridization reactions can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. Sequences that correspond or align more closely to the invention disclosed herein are comparably more preferred. Generally, a polynucleotide region of about 25 residues is essentially identical to another region, if the sequences are at least about 80% identical; more preferably, they are at least about 90% identical; more preferably, they are at least about 95% identical; still more preferably, the sequences are 100% identical. A polynucleotide region of 40 residues or more will be essentially identical to another region, after alignment of homologous portions if the sequences are at least about 75% identical; more preferably, they are at least about 80% identical; more preferably, they are at least about 85% identical; even more preferably, they are at least about 90% identical; still more preferably, the sequences are 100% identical.

In determining whether polynucleotide sequences are essentially identical, a sequence that preserves the functionality of the polynucleotide with which it is being compared is particularly preferred. Functionality can be determined by different parameters. For

example, if the polynucleotide is to be used in reactions that involve hybridizing with another polynucleotide, then preferred sequences are those which hybridize to the same target under similar conditions. In general, the T_m of a DNA duplex decreases by about 10°C for every 1% decrease in sequence identity for duplexes of 200 or more residues; or by about 50°C for duplexes of less than 40 residues, depending on the position of the mismatched residues (see, e.g., Meinkoth et al.). Essentially identical or equivalent sequences of about 100 residues will generally form a stable duplex with each other's respective complementary sequence at about 20°C less than T_m; preferably, they will form a stable duplex at about 15°C less; more preferably, they will form a stable duplex at about 10°C less; even more preferably, they will form a stable duplex at about 5°C less; still more preferably, they will form a stable duplex at about T_m. In another example, if the polypeptide encoded by the polynucleotide is an important part of its functionality, then preferred sequences are those which encode identical or essentially identical polypeptides. Thus, nucleotide differences which cause a conservative amino acid substitution are preferred over those which cause a non-conservative substitution, nucleotide differences which do not alter the amino acid sequence are more preferred, while identical nucleotides are even more preferred. Insertions or deletions in the polynucleotide that result in insertions or deletions in the polypeptide are preferred over those that result in the down-stream coding region being rendered out of phase; polynucleotide sequences comprising no insertions or deletions are even more preferred. The relative importance of hybridization properties and the encoded polypeptide sequence of a polynucleotide depends on the application of the invention.

A polynucleotide has the same characteristics or is the equivalent of another polynucleotide if both are capable of forming a stable duplex with a particular third polynucleotide under similar conditions of maximal stringency. Preferably, in addition to similar hybridization properties, the polynucleotides also encode essentially identical polypeptides.

"Conserved" residues of a polynucleotide sequence are those residues which occur unaltered in the same position of two or more related sequences being compared. Residues that are relatively conserved are those that are conserved amongst more related sequences than residues appearing elsewhere in the sequences.

"Related" polynucleotides are polynucleotides that share a significant proportion of identical residues.

As used herein, a "degenerate" oligonucleotide sequence is a designed sequence derived from at least two related originating polynucleotide sequences as follows: the residues that are conserved in the originating sequences are preserved in the degenerate sequence, while residues that are not conserved in the originating sequences may be provided as several alternatives in the degenerate sequence. For example, the degenerate sequence AYASA may be designed from originating sequences ATACA and ACAGA, where Y is C or T and S is C or G. Y and S are examples of "ambiguous" residues. A degenerate segment is a segment of a polynucleotide containing a degenerate sequence.

It is understood that a synthetic oligonucleotide comprising a degenerate sequence is actually a mixture of closely related oligonucleotides sharing an identical sequence, except at the ambiguous positions. Such an oligonucleotide is usually synthesized as a mixture of all possible combinations of nucleotides at the ambiguous positions. Each of the oligonucleotides in the mixture is referred to as an "alternative form".

A polynucleotide "fragment" or "insert" as used herein generally represents a sub-region of the full-length form, but the entire full-length polynucleotide may also be included.

Different polynucleotides "correspond" to each other if one is ultimately derived from another. For example, messenger RNA corresponds to the gene from which it is transcribed. cDNA corresponds to the RNA from which it has been produced, such as by a reverse transcription reaction, or by chemical synthesis of a DNA based upon knowledge of the RNA sequence. cDNA also corresponds to the gene that encodes the RNA. Polynucleotides also "correspond" to each other if they serve a similar function, such as encoding a related polypeptide, in different species, strains or variants that are being compared.

A "probe" when used in the context of polynucleotide manipulation refers to an oligonucleotide which is provided as a reagent to detect a target potentially present in a sample of interest by hybridizing with the target. Usually, a probe will comprise a label or a means by which a label can be attached, either before or subsequent to the hybridization reaction. Suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes.

A "primer" is an oligonucleotide, generally with a free 3' -OH group, that binds to a target potentially present in a sample of interest by hybridizing with the target, and thereafter promotes polymerization of a polynucleotide complementary to the target.

Processes of producing replicate copies of the same polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "amplification" or "replication". For example, single or double-stranded DNA may be replicated to form another DNA with the same sequence. RNA may be replicated, for example, by an RNA-directed RNA polymerase, or by reverse-transcribing the DNA and then performing a PCR. In the latter case, the amplified copy of the RNA is a DNA with the identical sequence.

A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using one or more primers, and a catalyst of polymerization, such as a reverse transcriptase or a DNA polymerase, and particularly a thermally stable polymerase enzyme. Generally, a PCR involves reiteratively forming three steps: "annealing", in which the temperature is adjusted such that oligonucletide primers are permitted to form a duplex with the polynucleotide to be amplified; "elongating", in which the temperature is adjusted such that oligonucleotides that have formed a duplex are elongated with a DNA polymerase, using the polynucleotide to which are formed the duplex as a template; and "melting", in which the temperature is adjusted such that the polynucleotide and elongated oligonucleotides dissociate. The cycle is then repeated until the desired amount of amplified polynucleotide is obtained. Methods for PCR are taught in U.S. Patent Nos. 4,683,195 (Mullis) and 4,683,202 (Mullis et al.).

As used herein, the term "operatively linked" means that the DNA molecule is positioned relative to the necessary regulation sequences, e.g., a promoter or enhancer, such that a promoter will direct transcription of RNA off the DNA molecule in a stable or transient manner. Regulatory elements include, but are not limited to promoter regions, enhancer regions, repressor binding regions, transcription initiation sites, ribosome binding sites, translation initiation sites, protein encoding regions, introns and exons, and termination sites for transcription and translation.

A "subject," "individual" or "patient" is used interchangeably herein, which refers to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the purpose of the experiment is to determine a correlation of an altered expression level of a gene with a particular type of cancer, it is generally preferable to use a positive control (a subject or a sample from a subject, carrying such alteration and exhibiting syndromes characteristic of that disease), and a negative control (a subject or a sample from a subject lacking the altered expression and clinical syndrome of that disease).

A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or polynucleotide sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the

polynucleotide comprising the viral genome or part thereof, and a therapeutic gene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. (see, e.g., WO 9527071) Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (see, WO 95/00655; WO 95/11984). Wild-type AAV has high infectivity and specificity integrating into the host cells genome. (Hermonat and Muzyczka (1984) *PNAS USA* **81**:6466; and Lebkowski et al. (1988) *Mol. Cell. Biol.* **8**:3988).

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, and targeted viral protein DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens.

Polynucleotides are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, an oligonucleotide containing a termination codon and an appropriate

restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

"Host cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous polynucleotides, polypeptides and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be procaryotic or eucaryotic, and include but are not limited to bacterial cells, yeast cells, plant cells, insect cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eucaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook, et al. (1989) *supra*). Similarly, an eucaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described below for constructing vectors in general.

As used herein "purified" or "isolated" shall mean not in the native or endogenous environment. In one embodiment, it shall mean removed from constituents normally

associated with the nucleic acid, vector, cell, protein or polypeptide, in its native or naturally occurring environment. The polynucleotides and/or proteins may be used in purified form when in the naturally occurring form. However, the polynucleotides and/or proteins need not be utilized in the "isolated" or "purified" state when present in an unnatural form, e.g., produced from a bacterial cell.

As used herein, "solid phase support" is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads. alumina gels. A suitable solid phase support may be selected on the basis of desired end use and suitability for various synthetic protocols. For example, for peptide synthesis, solid phase support may refer to resins such as polystyrene (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California). In a preferred embodiment for peptide synthesis, solid phase support refers to polydimethylacrylamide resin.

An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins, humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

An "antibody complex" is the combination of antibody (as defined above) and its binding partner or ligand.

An "antisense" copy of a particular polynucleotide refers to a complementary sequence that is capable of hydrogen bonding to the polynucleotide and can therefor, be capable of modulating expression of the polynucleotide. These may be DNA, RNA or analogs thereof, including analogs having altered backbones, as described above. The polynucleotide to which the antisense copy binds may be in singe-stranded form or in double-stranded form.

This invention provides a method for inhibiting a proinflammatory response in a suitable cell mixture, the method comprising administrating to the mixture an effective amount of an immunosuppressive agent which suppresses the proinflammatory activity of FasL. The immunosuppressive agent can be any agent which inhibits the immunostimulatory function of FasL. Such agents include, but are not limited to, anti-sense molecules that inhibit endogenous FasL expression, soluble Fas receptors or variants thereof, ribozymes that inhibit the endogeneous expression of FasL, drugs that inhibit FasL signalling, agents that induce the endogenous expression of TGF-β, such as cyclosporin or thrombospondin 1, (see Crawford, S.E. et al. (1998) *Cell* **93(7)**:1159), polynucleotides coding for an immunosuppressive agent such as TGF-β, or TGF-β protein or polypeptide.

It is to be understood, although not always explicitly stated that the above agents, molecules and compositions can be administered alone or in combination with a carrier, e.g., a pharmaceutically acceptable carrier.

It is a further aspect of this invention to provide a method for identifying agents which In one embodiment, the method further comprises coadministering an effective amount of FasL. The method can be practiced *in vitro*, *ex vivo* or *in vivo*.

As used herein, the term "proinflammatory response" shall mean an inflammatory response mediated by the Fas-FasL system, or a response which includes both cellular and humoral immunity , or in which an inflammatory response is mediated by neutrophils or cells of granulocytic or monocytic origin.

As used herein, the term "administration" shall mean providing to cells in *vitro* or *ex vivo*, an effective amount of FasL and/or the immunosuppressive agent. It also shall mean providing to cells in a subject *in vivo* an effective amount of FasL and/or the immunosuppressive agent. It also includes administering to cells *in vitro*, *ex vivo* or in a subject *in vivo*, an agent which inhibits the endogenous production of an effective amount of FasL and/or the production of an effective amount of the immunosuppressive agent by the cells *in vitro*, *ex vivo* or *in vivo*.

Thus, in one embodiment, the cell mixture comprises neutrophils as exemplified *in vitro* and *in vivo*, below. In a separate embodiment, the cell mixture comprises cells lacking FasL.

In one embodiment, the method is practiced by administration of a polynucleotide coding for FasL or soluble FasL. The term "FasL" is intended to include, but not be limited to the polynucleotide comprising the open reading frame of the FasL polynucleotide shown in Figures 4A and 5 and those polynucleotides coding for polypeptides and proteins having FasL biological activity. The method can be practiced by administering a polynucleotide coding for and expressing FasL or soluble FasL. The term "FasL" is intended to include, but not be limited to the polynucleotide comprising the open reading frame of the FasL polynucleotide provided in WO 98/03648, WO 97/33617, WO 95/18819, WO 95/13293; their complements; polynucleotides that hybridize to these sequences or their complements under stringent hybridization conditions; or those which are greater than 75%, more preferably greater than 85% and most preferably, greater than 90% homologous as determined by a sequence allignment program such BLAST and using the default search parameters with a cutoff of a high score of 50 or above, 100 or above, or more preferably, 150 or above (see http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html). It further includes polynucleotides coding for polypeptides having the sequence shown in SEQ ID NO 2 and/or 6, biologically equivalent mutants, analogs and variants thereof, such as those disclosed in WO 98/03648, WO/9733617, WO 95/18819, WO 95/13293. One of skill in the art can determine if a putative equivalent has the requisite biological activity by assaying the polynucleotide in the methods provided below. Although biological activity greater than the FasL polynucleotide provided herein is preferred, polynucleotides coding for polypeptides having less potent biological activity are useful as controls in the assays provided herein.

The term also is intended to include polynucleotides which correspond to the sequence shown in Figure 5, its complement, essentially identical polynucleotides or equivalent polynucleotides. The term "FasL" further includes the polynucleotides coding for ligand proteins of the TNF/nerve growth factor receptor family as shown in Figure 6 (SEQ ID NOS: 7 to 10), which includes two TNF receptors (type I, β or 55kD; type II, α or 75 kD), the low-affinity nerve growth factor receptor (CD40, CD27, CD30 and OX40), biologically equivalent mutants, analogs and variants thereof. It further includes polynucleotides coding for polypeptides having the sequence shown in SEQ ID NOS. 6 to 10 , biologically equivalent mutants, analogs and variants thereof. One of skill in the art

can determine if a putative equivalent has the requisite biological activity by assaying the polynucleotide in the methods provided below. Although biological activity greater than the FasL polynucleotide provided herein is preferred, polynucleotide coding for polypeptides having less potent biological activity are useful as controls in the assays provided herein.

It also includes polynucleotide sequences that encodes biologically equivalent proteins such as amino acids as shown in Figures 4 to 6, modified by having conservative amino acid substitutions.

The term also includes a polynucleotide coding for soluble FasL, the polynucleotide and amino acid sequence of which is shown in Figure 5 (SEQ ID NO: 5 and 6) and disclosed in WO 95/13701 and their complements; as well as polynucleotides that hybridize to these sequences, or their complements under stringent hybridization conditions or those polynucleotides which are greater than 75%, more preferably greater than 85% and most preferably, greater than 90% homologous as determined by a sequence allignment program such as BLAST and using the default search parameters with a cutoff of a high score of 50 or above, 100 or above, or more preferably 150 or above (see http://www.ncbi.nlm.nih.gov/BLAST/blast_ help.html). It also includes polynucleotides which correspond to the sequence shown in Figure 5, its complement, an essentially identical polynucleotide or an equivalent polynucleotide. It further includes a polynucleotide sequences that encodes biologically equivalent proteins such as amino acids as shown in Figure 5 and modified by having conservative amino acid substitutions. It further includes polynucleotides coding for polypeptides having the sequence shown in SEQ ID NO 6 or disclosed in WO 95/13701, biologically equivalent mutants, analogs and variants thereof. One of skill in the art can determine if a putative equivalent has the requisite biological activity by assaying the polynucleotide in the methods provided below. Although biological activity greater than the FasL polynucleotide provided herein is preferred, polynucleotide coding for polypeptides having less potent biological activity are useful as controls in the assays provided herein.

Each of the above noted compositions provides a separate embodiment of the invention, which may be practiced separately or in combination with each other or another, yet undetermined composition

This invention further includes the polynucleotides coding for FasL and soluble FasL as defined herein operatively linked to regulatory sequences required for transcription and/or translation of the polynucleotide in a host cell. In a further embodiment, the polynucleotides and regulatory sequences are administered in a gene delivery vehicle such as a liposome, a viral vector, or a plasmid. The host cell containing the FasL polynucleotide and soluble FasL polynucleotide also is provided by this invention. The host cell may be a procaryotic cell, such a bacterial cell or a eucaryotic cell such as an insect cell, a plant cell or an animal cell, such as a mammalian cell. When the host cells are maintained in culture, the cell culture is useful for the recombinant production of FasL and soluble FasL polypeptide or protein. Isolated, recombinantly produced FasL and soluble FasL polypeptide or protein are further provided by this invention.

As used herein, the term "FasL" also includes FasL protein and soluble FasL polypeptide, the amino acid sequences of which are provided in Figures 4 through 6, as well as analogs, muteins and variants thereof having comparable biological activity to wild-type FasL. The analogs, muteins and variants thereof can be assayed in the screening method described below to determine if they have the required biological activity.

The method also requires the administration of an effective amount of an immunosuppressive agent. As used herein, the term "immunosuppressive agent" is to include any agent which counteracts the proinflammatory response mediated by the sole administration of FasL. In one embodiment, the agent is a cytokine, such as TGF-β, the nucleotide for which is provided in Figure 7 and U.S. Patent Nos. 4,886,747; 5,168,051; 5,284,763; and 5,482,851. In a further embodiment, the TGF-β is any one of TGF-β 1-5. In a further embodiment, it includes a cytokine, e.g., an interferon or a growth factor. In a further embodiment, it is an antisense FasL polynucleotide or ribozyme which inhibits expression of endogeneous FasL, Fas soluble receptor protein or polynucleotide,or a drug that inhibits Fas signalling. In a further embodiment, it is an agent or compound which promotes the endogenous production of the immunosuppressive agent such as cyclosporin or thrombospondin 1, or stimulates its signal translation pathway. Finally, a further embodiment includes any combination of agents that stimulate Fas signalling and TGF-β signalling concurrently.

The term "TGF-β polynucleotide" is to include polynucleotides which correspond to the sequence shown in Figure 7 or U.S. Patent Nos. 4,886,747; 5,168,051; 5,284,763; and 5,482,851, their complements, an essentially identical polynucleotide or an equivalent polynucleotide. It further includes polynucleotides coding for polypeptides having the sequence shown in U.S. Patent Nos. 4,886,747; 5,168,051; 5,284,763; and 5,482,851, biologically equivalent mutants, analogs and variants thereof. One of skill in the art can determine if a putative equivalent has the requisite biological activity by assaying the polynucleotide in the methods provided below. Although biological activity greater than the TGF-β polynucleotide provided herein is preferred. polynucleotide coding for polypeptides having less potent biological activity are useful as controls in the assays provided herein.

It also includes polynucleotide sequences that encodes biologically equivalent proteins such as amino acids as shown in U.S. Patent Nos. 4,886,747; 5,168,051; 5,284,763; and 5,482,851 and those having conservative amino acid substitutions.

Each of the above noted compositions provides a separate embodiment of the invention, which may be practiced separately or in combination with each other or another, yet undetermined composition.

This invention further includes the polynucleotides coding for the immunosuppressive agent such as TGF-β as defined herein operatively linked to regulatory sequences for transcription and/or translation of the nucleic acid in a host cell. In some aspects, the polynucleotide and regulatory sequences are administered in gene delivery vehicle such as a liposome, a viral vector, or a plasmid. The host cell containing the immunosuppressive polynucleotide also is provided by this invention. The host cell may be a procaryotic cell, such a bacterial cell or a eucaryotic cell such as an insect cell, a plant cell or an animal cell, such as a mammalian cell. When the host cells are maintained in culture, the cell culture is useful for the recombinant production of immunosuppressive polypeptide or protein. Isolated, recombinantly produced TGF-β polypeptide or protein are further provided by this invention.

As used herein, the term "immunosuppressive agent" also includes TGF-β protein and polypeptide, the amino acid sequences of which are provided in U.S. Patent Nos. 4,886,747; 5,168,051; 5,284,763; and 5,482,851 as well as analogs, muteins and variants

thereof having comparable biological activity to wild-type TGF-β. The analogs, muteins and variants thereof can be assayed and determined in the screening method described below to determine if they have the required biological activity. TGF-β1 protein also is commercially available from R&D Systems, Minneapolis, MN.

This invention further provides a method to inhibit the proinflammatory response mediated by FasL comprising administering to a cell mixture an effective amount of TGF-β1-5, analogs, mutants and variants thereof.

When the methods indicated above are practiced in vivo in an animal model or subject, they are useful to ameliorate the symptoms of diseases associated with an undesired FasL-mediated proinflammatory response, e.g., as graft versus host disease, an autoimmune disease, such as systemic lupus erythrematosus, rheumatoid arthritis, and psoriasis. Thus, this invention also provides a method for treating an undesired FasL-mediated proinflammatory response. This includes, but is not limited to conditions such as graft versus host disease, an autoimmune disease, such as systemic lupus erythrematosus, rheumatoid arthritis, and psoriasis. The method requires administering to the subject an effective amount an immunosuppressive agent, under conditions favorable for the treatment of the an undesired FasL-mediated proinflammatory response. In a further embodiment, an effective amount of FasL also is administered. As indicated above, in one aspect, this method comprises administration of an agent that stimulates endogenous production of an effective amoount of FasL and/or an immunosuppressive agent in a subject. In a further aspect, the method comprises administration of an agent such as cyclosporin A, that stimulates the endogenous production of an immunosuppressive agent, e.g., TGF-β, in a subject. In a further embodiment, the invention is practiced with an agent that stimulates the Fas-signal transduction pathway and/or inhibits the relevant elements of the TGF-β signal transduction pathway, e.g., those agents that inhibit p38 MAP kinase in neutrophils (See Figures 8A and 8B, above).

Gene Therapy

When the FasL polynucleotide and/or immunosuppressive agent is administered to the cells *in vitro,* any suitable gene transfer method is intended. Such methods include, but are not limited to by electroporation, transformation or transfection procedures. Sambrook

et al. (1989) *supra*. Alternatively, the gene is inserted into an appropriate expression vector by methods well known in the art and as described below. FasL and the immunosuppressive agent can be administered in separate gene delivery vehicles or combined into one gene delivery vehicle.

In one aspect, polynucleotides encoding one or more of the embodiments of FasL and/or immunosuppressive agent can be delivered to the subject using a gene delivery vehicle. The methods of this invention are intended to encompass any method of gene transfer into the subject. Examples of delivery mechanisms include, but are not limited to viral mediated gene transfer, liposome mediated transfer, transformation, transfection and transduction, *e.g.*, viral mediated gene transfer such as the use of vectors based on DNA viruses such as adenovirus, adeno-associated virus and herpes virus, as well as retroviral based vectors.

Vectors Useful in Genetic Modifications

In general, genetic modifications of cells *in vitro, ex vivo* and *in vivo*, employed in the present invention are accomplished by introducing a vector containing a polynucleotide as described herein. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used. Viral vectors useful in the genetic modifications of this invention include, but are not limited to adenovirus, adeno-associated virus vectors, retroviral vectors and adeno-retroviral chimeric vectors.

Construction of Recombinant Adenoviral Vectors or Adeno-Associated Virus Vectors

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (see, *e.g.,* Karlsson, et al. (1986) *EMBO J.* **5**:2377; Carter (1992) *Current Opinion in Biotechnology* **3**:533; Muzcyzka (1992) *Current Top. Microbiol. Immunol.* **158**:97; and GENE TARGETING: A PRACTICAL APPROACH (1992) (A. L. Joyner ed., Oxford University Press, NY). Several different approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus system.

The recombinant adenoviral vectors based on the human adenovirus 5 ((1988) *Virology* **163**:614) are missing essential early genes from the adenoviral genome (usually

E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products *in trans*. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in cells infected with the replication deficient adenovirus. Although adenovirus-based gene transfer does not result in integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and transfect non-replicating cells. Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines and are commercially available from the ATCC. However, other cell lines which allow replication-deficient adenoviral vectors to propagate therein can be used, including HeLa cells.

Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B. et al. (eds.) VIROLOGY, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F. et al. pp. 109-128 in METHODS IN MOLECULAR BIOLOGY, Vol. 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N. et al. (1995) *FASEB Journal* **9**:190; Schreier, H. (1994) *Pharmaceutica Acta Helvetiae* **68**:145; Schneider and French (1993) *Circulation* **88**:1937; Curiel D.T. et al.(1992) *Human Gene Therapy* **3**:147; Graham, F.L. et al. WO 95/00655 (5 January 1995); Falck-Pedersen, E.S. WO 95/16772 (22 June 1995); Denefle, P. et al. WO 95/23867 (8 September 1995); Haddada, H. et al. WO 94/26914 (24 November 1994); Perricaudet, M. et al. WO 95/02697 (26 January 1995); and Zhang, W. et al. WO 95/25071 (12 October 1995). A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996). See also, the papers by Vile et al. (1997) *Nature Biotechnology* **15**:840; and Feng et al.(1997) *Nature Biotechnology,* **15**:866, describing the construction and use of adeno-retroviral chimeric vectors that can be employed for genetic modifications.

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Carter, B. HANDBOOK OF PARVOVIRUSES, Vol. **I**, pp. 169-228, 1990; Berns, VIROLOGY, pp. 1743-1764 (Raven Press 1990); Carter,

B. (1992) *Curr. Opin. Biotechnol.* **3**:533; Muzyczka, N. (1992) *Current Topics in Micro and Immunol*, **158**:92; Flotte, T.R. et al. (1992) *Am. J. Respir. Cell Mol. Biol.* **7**:349; Chatterjee et al. (1995) *Ann. NY Acad. Sci.* **770**:79; Flotte, T.R. et al. WO 95/13365 (18 May 1995); Trempe, J.P. et al. WO 95/13392 (18 May 1995); Kotin, R. (1994) *Human Gene Therapy* **5**:793; Flotte, T.R. et al. (1995) *Gene Therapy* **2**:357; Allen, J.M., WO 96/17947 (13 June 1996); and Du et al. (1996) *Gene Therapy* **3**:254.

Construction of Retroviral Vectors

Retroviral vectors useful in the methods of this invention are produced recombinantly by procedures already taught in the art. For example, WO 94/29438 describes the construction of retroviral packaging plasmids and packaging cell lines. As is apparent to the skilled artisan, the retroviral vectors useful in the methods of this invention are capable of infecting the cells described herein. The techniques used to construct vectors, and transfix and infect cells are widely practiced in the art. Examples of retroviral vectors are those derived from murine, avian or primate retroviruses. Retroviral vectors based on the Moloney murine leukemia virus (MoMLV) are the most commonly used because of the availability of retroviral variants that efficiently infect human cells. Other suitable vectors include those based on the Gibbon Ape Leukemia Virus (GALV) or HIV.

In producing retroviral vector constructs derived from the Moloney murine leukemia virus (MoMLV), in most cases, the viral gag, pol and env sequences are removed from the virus, creating room for insertion of foreign DNA sequences. Genes encoded by the foreign DNA are usually expressed under the control of the strong viral promoter in the LTR. Such a construct can be packed into viral particles efficiently if the gag, pol and env functions are provided *in trans* by a packaging cell line. Thus, when the vector construct is introduced into the packaging cell, the gag-pol and env proteins produced by the cell, assemble with the vector RNA to produce infectious virions that are secreted into the culture medium. The virus thus produced can infect and integrate into the DNA of the target cell, but does not produce infectious viral particles since it is lacking essential packaging sequences. Most of the packaging cell lines currently in use have been transfected with separate plasmids, each containing one of the necessary coding sequences, so that multiple recombination events are necessary before a replication competent virus

can be produced. Alternatively, the packaging cell line harbors an integrated provirus. The provirus has been crippled so that, although it produces all the proteins required to assemble infectious viruses, its own RNA cannot be packaged into virus. Instead, RNA produced from the recombinant virus is packaged. The virus stock released from the packaging cells thus contains only recombinant virus.

The range of host cells that may be infected by a retrovirus or retroviral vector is determined by the viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the transduced cell and the stable production of the foreign gene product. In general, murine ecotropic env of MoMLV allows infection of rodent cells, whereas amphotropic env allows infection of rodent, avian and some primate cells, including human cells. Amphotropic packaging cell lines for use with MoMLV systems are known in the art and commercially available and include, but are not limited to, PA12 and PA317. Miller et al. (1985) *Mol. Cell. Biol.* **5**:431; Miller et al. (1986) *Mol. Cell. Biol.* **6**:2895; and Danos et al. (1988) *Proc. Natl. Acad. Sci. USA* **85**:6460. Xenotropic vector systems exist which also allow infection of human cells.

The host range of retroviral vectors has been altered by substituting the env protein of the base virus with that of a second virus. The resulting, "pseudotyped", virus has the host range of the virus donating the envelope protein and expressed by the packaging cell line. Recently, the G-glycoprotein from vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. Burns, et al. (1993) *Proc. Natl. Acad. Sci USA* **90**:8033; and PCT patent application WO 92/14829. Since infection is not dependent on a specific receptor, VSV-G pseudotyped vectors have a broad host range.

Usually, the vectors will contain at least two heterologous genes or gene sequences: (i) the therapeutic gene to be transferred; and (ii) a marker gene that enables tracking of infected cells. As used herein, "therapeutic gene" can be an entire gene or only the functionally active fragment of the gene capable of compensating for the deficiency in the patient that arises from the defective endogenous gene. Therapeutic gene also encompasses antisense oligonucleotides or genes useful for antisense suppression and ribozymes for ribozyme-mediated therapy. For example, in the present invention, a

therapeutic gene may be one that neutralizes the immunosuppressive factor or counter its effects.

The method also can be practiced *ex vivo* using a modification of the method described in Lum et al. (1993) *Bone Marrow Transplantation* **12**:565 or a modification of the method described in U.S. Patent No. 5,399,346. Generally, a sample of cells such as bone marrow cells or MLC containing neoplastic cells can be removed from a subject or animal using methods well known to those of skill in the art. An effective amount of FasL nucleic acid and/or immunosuppressive agent is added to the cells and the cells are cultured under conditions that favor internalization of the nucleic acid by the cells. The transformed cells are then returned or reintroduced to the same subject or animal (autologous) or one of the same species (allogeneic) in an effective amount and in combination with appropriate pharmaceutical compositions and carriers.

Non-viral vectors, such as plasmid vectors useful in the genetic modifications of this invention, can be produced according to methods taught in the art. References describing the construction of non-viral vectors include the following: Ledley, F.D. (1995) *Human Gene Therapy* **6**:1129; Miller, N. et al. (1995) *FASEB Journal* **9**:190; Chonn, A. et al. (1995) *Curr. Opin. in Biotech.* **6**:698; Schofield, J.P. et al. (1995) *British Med. Bull.* **51**:56, Brigham, K.L. et al. (1993) *J. Liposome Res.* **3**:31; Brigham, K.L. WO 9106309 (16 May 1991); Felgner, P.L. et al. WO 9117424 (14 November 1991); Solodin, et al. (1995) *Biochemistry* **34**:13537; WO 9319768 (14 October 1993); Debs, et al. WO 9325673; Felgner, P.L. et al. U.S. Patent 5,264,618 (November 23, 1993); Epand, R.M. et al. U.S. Patent 5,283,185 (February 1, 1994); Gebeyehu, et al. U.S. Patent 5,334,761 (August 2, 1994); Felgner, P.L. et al. U.S. Patent 5,459,127 (October 17, 1995); Overell, R.W. et al. WO 9528494 (26 October 1995); Jessee, WO 9502698 (26 January 1995); Haces and Ciccarone, WO 9517373 (29 June 1995); and Lin, et al. WO 9601840 (25 January 1996).

More than one gene can be administered per vector or alternatively, more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the vector can include the regulatory and untranslated sequences. For gene therapy in human patients, the polynucleotides encoding the FasL, soluble FasL and/or the immunosuppressive agent will generally be of human origin although genes from other closely related species that exhibit high homology and biologically identical or equivalent

function in humans may be used, if the gene product does not induce an adverse immune reaction in the recipient.

Assessing Efficacy of Gene Transfer *In vitro* or *In vivo*

The efficacy of gene transfer into the cells *in vitro* or in *vivo* can be monitored by any method known in the art. For example, as described above, a reporter or marker gene can be included in the gene delivery vehicle to facilitate identification of those cells into which the vehicle is successfully incorporated (Kass-Eisler et al. (1994) *Gene Therapy* **1**:395). Especially in the *in vitro* and *ex vivo* contexts, marker genes may prove especially helpful. Screening markers or reporter genes are genes that encode a product that can readily be assayed. Non-limiting examples of screening markers include genes encoding for green fluorescent protein (GFP) or genes encoding for a modified fluorescent protein.

Preferably, the marker gene included in the delivery vehicle is a selectable marker. A "positive" selectable marker gene encodes a product that enables only the cells that carry the gene to survive and/or grow under certain conditions. For example, plant and animal cells that express the introduced neomycin resistance (Neo^r) gene are resistant to the compound G418. Cells that do not carry the Neo^r gene marker are killed by G418. Negative selectable marker genes encode a product that allows cells expressing that product to be selectively killed. For example, as described above the conditionally activated cytotoxic agent may also be a selectable marker such as HSV-tk. Cells expressing this gene can be selectively killed using gancyclovir or acyclovir.

Other methods that can be employed to determine the extent of gene transfer include:

(1) quantitation of vector specific DNA by PCR;

(2) quantitation of transgene specific mRNA by RT-PCR;

(3) quantitation of the amount of soluble FasL and/or TGF-β in the serum by ELISA;

(4) quantitation of the amount of anti-FasL and/or immunosuppressive antibody in the serum by ELISA; and

(5) quantitation of the amount of FasL and/or TGF-β biological activity within the target tissue by homogenization of the tissue and assaying for enzymatic activity or

treating the tissue with a suitable substrate that will be converted by the FasL and/or TGF-β into a compound that can be quantitated by colorimetric methods.

Protein Therapy

The FasL proteins and polypeptides and immunosuppressive agents, *e.g.*, TGF-β, of this invention are obtainable by a number of processes well known to those of skill in the art, which include purification, chemical synthesis and recombinant methods. TGF-β and other immunosuppressive agents are commercially available. Full length FasL protein can be purified from a $FasL^+$ cell or tissue lysate and TGF-β from tissues using the process described below or by methods such as immunoprecipitation with antibody, and standard techniques such as gel filtration, ion-exchange, reversed-phase, and affinity chromatography using a fusion protein as shown herein. For such methodology, see for example Deutscher et al. GUIDE TO PROTEIN PURIFICATION: METHODS IN ENZYMOLOGY Vol. 182 (Academic Press (1990)). Accordingly, this invention also provides the processes for obtaining the proteins and polypeptides useful this invention as well as the products obtainable and obtained by these processes.

Compositions containing the polypeptides and proteins for use in this invention, including compositions comprising a pharmaceutically acceptable carrier, are further provided by this invention. This invention further provides use of the polynucleotides and/or proteins disclosed herein for the manufacture of medicaments to inhibit a proinflammatory response.

The proteins and polypeptides also can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Applied Biosystems, Inc./Perkin Elmer, Model 430A or 431A, Foster City, CA and the amino acid sequences provided in Figures 4 through 6. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Accordingly, this invention also provides a process for chemically synthesizing the proteins of this invention by providing the sequence of the protein and reagents, such as amino acids and enzymes and linking together the amino acids in the proper orientation and linear sequence.

Alternatively, the proteins and polypeptides can be obtained by well-known recombinant methods as described, for example, in Sambrook et al. (1989) *supra*, using the host cell and vector systems described and exemplified below. This invention further provides a process for producing an immunosuppressive protein such as TGF-β and FasL protein, analog, mutein or fragment thereof, by growing a host cell containing a polynucleotide encoding the mammalian protein, the polynucleotide being operatively linked to a promoter of RNA transcription. The host cell is grown under suitable conditions such that the polynucleotide is transcribed and translated into protein and purifying the protein so produced.

Screening Assay

The potentially therapeutic agent identified by the method of this invention is an agent that is an agonist or antagonist of FasL stimulation of a proinflammatory response. In one aspect the method comprises (a) contacting a target cell mixture and a control cell mixture with FasL and an immunosuppressive agent; (b) contacting the target cell mixture with a candidate therapeutic agent; (c) assaying the target cell mixture for localized immune response; and (d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates FasL stimulation of the proinflammatory response.

In a separate aspect, the assaying of step (c) comprises assaying for modulation of a neutrophil mediated proinflammatory response or alternatively, assaying for modulation of a neutrophil mediated proinflammatory response, as indicated in the examples set forth below.

In a further aspect of the method the control cell mixture is admixed or contacted with an effective amount of FasL and an effective amount of an immunostimulatory agent subsequent to contacting the control cell mixture with the test agent. In a yet further aspect of the method, the control cell mixture is admixed or contacted with an effective amount of FasL and/or only an amount of an immunostimulatory agent subsequent to contacting the control cell mixture with the test agent.

For the purposes of this invention, an "agent" is intended to include, but not be limited to, a small organic molecule, a compound, a composition, a DNA molecule, an RNA molecule, a protein, a polypeptide, an antibody, an antibody fragment, an anti-idiotypic antibody or fragment or a fusion protein. It should be understood, although not

always explicitly stated that the agent is used alone or in combination with another agent, having the same or different biological activity as the agents identified by the inventive screen. The agents and methods are also intended to be combined with other therapies.

Thus, to practice the method *in vitro*, suitable cell cultures or tissue cultures are first provided. The cell is a cultured cell or alternatively, the cells can be from a tissue biopsy. The cells are cultured under conditions (temperature, growth or culture medium and gas (CO_2)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints.

As is apparent to one of skill in the art, suitable cells may be cultured in microtiter plates and several agents may be assayed at the same time by noting phenotypic changes or cell death.

As is known to those of skill in the art, a positive and negative control sample should be run simultaneously with the test sample. The positive control will receive the FasL polynucleotide or protein and/or the immunosuppressive polynucleotide or protein. The negative control will not receive FasL polynucleotide or protein nor the immunosuppressive polynucleotide/protein nor the test agent. The use of the negative control as compared to the test sample and the positive control allows one of skill in the art to compare the activity of the test agent versus the activity of the agent as against another agent having FasL and/or immunosuppressive activity. Each sammple must be assayed in the presence and absence of neutrophils to determine if the neutrophil-mediated response is inhibited. The identification of this activity allows one to find alternative therapies to the FasL polynucleotide or protein and/or other immunosuppressive agents.

The test agent is contacted with the cells under conditions which does not inhibit the proliferation or death of the cells. When the agent is a composition other than a DNA or RNA nucleic acid molecule, the suitable conditions may be directly adding the agent to the cell culture or added to culture medium. As is apparent to those skilled in the art, an "effective" amount must be added which can be empirically determined. The protein or polypeptide, is administered to the cells, *in vitro* or *in vivo*, as described above.

The test cell is grown in small multi-well plates and is used to detect the biologic activity of test agents. For the purposes of this invention, the successful candidate drug will promote (angonist) or block (antagonist) the immunosuppressive effect of an

immunosuppressive agent such as TGF-β1-5. If it the agent has no effect on the activity of the test cell sample but still stimulates the proinflammatory response, the agent is a candidate agent having FasL activity or immunostimulatory activity and is a possible substitute for FasL in the methods provided herein.

Kits containing the agents and instructions necessary to perform the screen and *in vitro* are further provided by this invention

The agents identified herein as effective for their intended purpose can be administered to subjects or individuals susceptible to or at risk of developing a disease correlated to the presence of an unwanted immune FasL-mediated immune response such as autoimmune disease or graft versus host disease. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. To determine patients that can be beneficially treated, a suitable cell sample is removed from the patient. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the agent. When delivered to an animal, the method is useful to further confirm efficacy of the agent.

One of skill in the art will know when the object of the method has been met by noting a reduction, inhibition or delay in the proinflammatory response. For example, a reduction of FasL-mediated cytotoxicity against T lymphocytes by chromium release assay is one means by which to monitor the response. Alternatively, one may note the reduction of the proinflammatory response *in vivo,* by noting the reduction in the severity of symptoms associated with a disease associated with this response such as graft versus host disease, an autoimmune disease, such as systemic lupus erythrematosus, rheumatoid arthritis, and psoriasis. The method may be practiced in an appropriate experimental animal model. For example, to determine their ability to treat rheumatoid arthritis, they may be assayed in collagen- or adjuvant-induced arthritis in rats. Reduction of disease severity in this model is measured by scales that respectively indicate degree of paralysis or joint swelling, as commonly used in the art. Survival after a defined time may also be an appropriate endpoint in certain animal models. Ability to treat diabetes can be modeled in the non-obese diabetic (NOD) mouse or BB rat. Numerous animal models are available

to test the treatment of these and other autoimmune diseases (see, European Patent 0304291 and references cited therein) or other inflammatory diseases (see, EP 94903357.5 and references cited therein).

The following experimental examples are intended to illustrate, but not limit the invention.

EXPERIMENTAL EXAMPLES

The cellular and molecular basis for the effect of FasL has been determined by the inventors. While it has been previously reported that neutrophils contribute to the FasL antitumor response (Arai, H. et al. (1997) *Proc. Natl. Acad. Sci USA* **94**:13862; Seino, K.-I. (1997) *Nat.Med.* **3**:165), the mechanism by which these cells promoted tumor rejection was not explained nor understood. To address this question, it was determined whether neutrophils could mediate lysis of CT26-FasL cells *in vitro*. Neutrophils enriched from human peripheral blood were incubated with CT26-FasL target cells or a $FasL^-$ negative control cell line, CT26-neo. Neutrophils lysed CT26-FasL cells specifically in a dose-dependent fashion, in contrast to CT26-neo cells (Figure 1A). Depletion of these effector cells with antibodies to T lymphocytes, NK cells, or macrophages did not reduce this cytolysis (Figure 1B). This effect was also examined with neutrophils derived from peripheral blood leukocytes of syngeneic mice. Such cells from Balb/c mice effectively lysed CT26-FasL tumor cells, in contrast to a control cell population depleted with a neutrophil-specific antibody, which did not retain this activity (Figure 1C). These results demonstrated that neutrophils interacted directly with $FasL^+$ cells to mediate their destruction.

To assess the role of FasL in neutrophil-mediated cytolysis, a Fas-Fc fusion protein was included in the cytotoxicity assay. Lysis of CT26-FasL cells was inhibited specifically by the Fas-Fc fusion protein but not by a negative control immunoglobulin (Figure 2A). Furthermore, bystander killing was observed when chromium-labeled CT26-neo were incubated together with unlabeled CT26-FasL cells (Figure 2B), suggesting that lysis of CT26-FasL cells was not due to intrinsic susceptibility of CT26-FasL cells but instead to the ability of FasL to induce neutrophil cytotoxic effector function locally.

Because CT26-FasL cells do not express Fas (Arai, H. et al. (1997) *Proc. Natl. Acad. Sci USA* **94**:13862), it is likely that inhibition by Fas-Fc was mediated by engagement of the neutrophil Fas receptor by FasL, which apparently promotes cytotoxic function. Because a bystander effect was observed in the presence of FasL, this cytotoxic effect may be a consequence of neutrophil degranulation.

The ability of different candidate cytokines to modulate neutrophil cytotoxicity was further analyzed. Recombinant TGF-β1 was found to effectively inhibit neutrophil-mediated lysis, in contrast to other relevant cytokines, for example, IL-10 and GM-CSF (Figure 3A). This effect was not related to neutrophil cell death since cell viability was unchanged during the incubation period (Figure 3, legend). In addition, TGF-β1 did not affect Fas expression or neutrophils or FasL expression or CT26-FasL cells. It also had no effect on the FasL-mediated lysis of Jurkat cells, a susceptible T-cell leukemia cell line (Figure 3B). This latter finding demonstrates that TGF-β does not block apoptosis in Fas^+ cells. Preincubation of neutrophils, but not target cells, with TGF-β1 substantially decreased cytolysis of CT26-FasL cells (Figure 3C), further suggesting that the inhibitory effect of TGF-β1 was achieved by its effect on neutrophil activation. To determine whether this effect was relevant to the lack of inflammation observed intraocularly, aqueous humor fluid was tested in this assay. Addition of this fluid to neutrophils, but not a heat-inactivated aliquot, inhibited their ability to lyse CT26-FasL cells (Figure 3D). This effect was reversed by addition of a neutralizing soluble TGF-β receptor protein (Figure 3E), suggesting that TGF-β was a major component of the aqueous humor that inhibits neutrophil-mediated cytolysis through its effect on cellular activation.

The mechanism by which $FasL^+$ cells evade destruction by neutrophils is central to an understanding of the generation of immune tolerance. While it has been postulated that FasL may provide immune protection, the results reported here suggest that additional local inhibitory factors likely regulate the inflammatory response. The role of FasL in the maintenance of immune privilege in the eye (Griffith, T. et al. (1995) *Science* **270**:1189) and testis (Bellgrau, D. et al. (1995) *Nature* **377**:630) is well-recognized. The correlation of corneal graft survival with FasL expression further supports this concept (Stuart, P.M. et al. (1997) *J Clin. Invest.* **99**:396); however, the proinflammatory effect of FasL has raised questions about the suppression by this protein and its contribution to graft survival. These

findings reported herein demonstrate that $FasL^+$ cells interact with neutrophils to promote the activation of cytolytic function and this effect is normally counteracted by a second immunosuppressive agent, such as TGF-β.

Previous studies have shown that corneas grafted to the eyes of allogeneic recipients are often accepted, in contrast to heterotropic corneal grafts to the skin which are rejected (Niederkorn, J.Y. (1990) *Transplantation* **50**:231). In addition, allogeneic heterotropic testicular tissue grafted to the anterior humoral chamber show prolonged survival, in contrast to the rapid rejection observed when this tissue was grafted to other organs (Turner, C.D. (1938) *The American Journal of Anatomy* **63**:101). TGF-β is known to be present in the aqueous humor (Wilbanks, G.A. and Streilein, W. (1992) *Eur. J. Immunol* **22**:103) and was previously suggested to play a role in immune tolerance through its effect on T cell proliferation (Li, H. et al. (1997) *Occul. Immunol. Inflamm.* **5**:75); however, TGF-β can also promote T cell survival in some instances, and its effect on innate immune responses mediated by neutrophils was previously unknown. It is interesting to note that TGF-β1 does not block FasL-induced apoptosis of a T leukemia line at the same time that it inhibited neutrophil function (Figure 3B). Taken together, these findings suggest that FasL, in combination with TGF-β, both suppresses inflammatory effects and promotes lymphocyte clonal deletion. Among current therapies for graft rejection, the immunosuppressive drug, cyclosporin A, has recently been found to stimulate TGF-β secretion (Shihab, F.S. (1996) *Semin. Nephrol.* **16**:536; Shin, G.T. et al. (1998) *Transplantation* **65**:313; Khanna, A. (1997) *Transplantation* **63**:1037; Zhang, X. et al. (1995) *J. Exp. Med.* **182**:699) and may, in part, work through this mechanism. Similarly, in malignancies resistant to immune recognition, both FasL and TGF-β1 have been detected in the tumor stroma (Ritvos, M. (1991) *Endocrinology* **129**:2240; Daughaday, W.H. and Deuel, T.F. (1991) *Endocrinol. Metab. Clin-North Am.* **20**:539). The data provided herein show that co-expression of such proteins in tumors would render immune therapies unsuccessful in the absence of strategies to neutralize or surmount their function.

Experimental Methods

Cells

Renca and CT26 were obtained from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, U.S.A., and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics.

Generation and expression of FasL using gene transfer

A mouse cDNA encoding FasL was synthesized from mouse spleen poly(A) RNA by reverse transcription (RT)-PCR using Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) and Expand™ long template PCR system (Boehringer Mannheim, Germany) (sense primer: AATTGTCGACCAC CATGGAGCAGCCCATGAATTACCATGTCCC, antisense primer: AATTGGATCCTCTAGATTAAAGCTTATACAAGCCGAAAAAGGT CTT). The FasL cDNA was inserted into the mammalian expression vector pVR1012 neo (Vical, Inc., San Diego, CA) harboring CMV enhancer/promoter, bovine growth hormone poly(A) signal[28] and neomycin resistant gene as a selection marker. CT26 cells were cultured in RPMI 1640 media (Gibco BRL) with 10% fetal calf serum (FCS). CT26 cells were transfected with linearized FasL/pVR1012 neo plasmid by electroporation using Gene pulser (BioRad, Hercules, CA), and transfected cells were selected with 1 mg/ml of Geneticin (Gibco BRL). A clone that expressed FasL at high levels (CT26-FasL) was isolated by limiting dilution.

Generation of stably transduced FasL-expressing cell line

The CT26 cell line which expresses FasL stably (CT26-FasL) was generated as previously reported in Arai, H. et al. (1997), *supra*. Briefly, the mouse FasL cDNA was inserted into a mammalian expression vector which utilizes the CMV enhancer/promoter, bovine growth hormone poly-A signal, and neomycin resistant gene as a selection marker. CT26 cells were transfected with this plasmid by electroporation and were selected with 1 mg/ml of Geneticin (GIBCO BRL, Gaithersburg, MD). A clone which expressed FasL at

high levels was isolated by limiting dilution. As a control, the CT26 clone transfected with the plasmid backbone (CT26-neo) was prepared in the same way.

Analysis of FasL, expression by immunostaining

Target cells (1 x 10^6) were stained with anti-Fas antibody (Pharmingen, San Diego, CA) or isotype control IgG followed by FITC-conjugated anti-IgG second antibody (Pharmingen), or Fas-Fc fusion protein (described below) followed by FITC-conjugated antibody to the Fc fragment of IgG (Jackson Immunoresearch Lab, West Grove, PA) to detect the expression of Fas or FasL, respectively. In all instances, conditioned medium from vector-control transfected 293 cells as used as a negative control. Relative fluorescence intensity was measured by FACS analysis. CT26 cells transfected with pVR1012 neo backbone (CT26-neo) were prepared for the control at the same time.

To construct the Fas-Fc fusion protein, cDNAs encoding the mouse Fas extracellular domain and the human IgG Fc portion[29] were synthesized by RT-PCR Fas sense primer: AATTGTCGACCACCATGGTGTGGATCTG GGCTGTCCTGCCTCTG, antisense primer: AATTGGATCCTCGAGGCGA TTTCTGGGACTTTGTTTCCTGCAG; IgG Fc sense primer: AATTCTGCAG CTCGAGCCCAAATCTTGTGACAAAACTCACACA, antisense primer: AATTGGATCCTCTAGATCATTTACCCGGAGACAGGGAGAGGCTCTT) and were connected at the XHoI site on the primer. The chimeric cDNA was inserted into pVR1012 neo backbone and transfected into 293 cells by the calcium-phosphate method. On day 7 post-transfection, the conditioned medium was collected, filtered and used in flow cytometric [fluorescence-activated cell sorting (FACS)] analysis.

Neutrophil and FasL-induced cytotoxicity assays

Neutrophil-mediated cytotoxicity was assayed as described previously (Martin, W.J. (1984) *Am. Rev. Resp. Dis.* **130**:209; Varani, J. et al. (1989) *Am. J. Path.* **135**:435; and Okrent, D.G. et al. (1990) *Am. Rev. Resp. Dis.* **141**:179) with modification. Briefly, CT26-FasL or CT26-neo cells were incubated for 2 hours at 37°C with 70°Ci of ^{51}Cr sodium chromate (Amersham,) in 100 µl of RPMI 1640 containing 10% FCS. The ^{51}Cr-labeled target cells (1x10^4) were mixed with human neutrophils (at the indicated

effector/target ratios) in fibronectin-coated plates (Collaborative Biomedical Product, Bedford, MA 01730) in a total volume of 200 µl in 0.5% FCS. The plates were centrifuged at 700 rpm for 3 minutes and incubated for 19 hours at 37°C in 5% CO_2/95% air. After centrifugation at 1000 rpm for 5 minutes, the supernatants were collected with the harvesting frame (Squadron, Sterling, VA) and assayed for radioactivity.

FasL-mediated cytotoxicity against T lymphocytes was assayed as described in Bellgrau, D. et al. (1995) *Nature* **377**:630. Jurkat cells ($1x10^6$) were incubated for 2 hours at 37°C with 70°Ci of ^{51}Cr sodium chromate (Amersham) in 100 µl of RPMI 1640 containing 10% FCS. The ^{51}Cr labeled target cells ($1x10^4$) were mixed with CT26-FasL ($1x10^5$) in a total volume of 200 µl for 4 hours and harvested as described above. Spontaneous release of ^{51}Cr was determined by incubating target cells with medium alone, whereas the maximum release was determined by incubating in 0.1% Triton X-100. The specific lysis (%) was calculated as: (experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(maximum ^{51}Cr release - spontaneous ^{51}Cr release).

Figure 1A shows neutrophil dose-dependent killing of $FasL^+$ CT26 cells. Specific killing of CT26-FasL cells, but not CT26-neo cells by human neutrophils was determined by a chromium release assay *in vitro* described above. CT26-FasL and CT26-neo target cells were labeled with ^{51}Cr and mixed with neutrophils at various ratios. The data shown represent the mean (± SD) of three experiments.

Figure 1B shows neutrophil effector-mediated killing. Human neutrophils were prepared as described below. Human T lymphocytes, NK cells, and macrophages were depicted from the isolated neutrophils by an immunomagnetic separation method (as described in Horgan, K. and Shaw, S. (1998) *Curr. Prot. Immu.* **7**:4) using anti-Fas3, anti-Fas65 and anti-Fas115/c (Phamingen, Neomarkers). The effector cells were mixed with FasL cells at a 50:1 ratio.

Figure 1C shows cytolysis mediated by neutrophils from syngeneic animals. Mouse neutrophils were prepared as described below. Neutrophils were mixed with FasL cells at a 50:1 ratio. Neutrophil-depleted mouse spleen cells were used as control. Neutrophils were depleted by using an anti-Ly6G (Pharmingen) as described in Figure 1B, above.

Adenoviral vectors

Recombinant adenoviral vectors, e.g., ADV-FasL as described herein, are preferred for the *in vivo* transfer of therapeutic polynucleotides. It can be prepared by homologous recombination between sub360 genomic DNA, an Ad5 derivative with a deletion in the E3 region, and a FasL expression plasmid, pAd-FasL. The pAd-FasL encodes the mouse FasL cDNA under control of the cytomegaloviral enhancer/promoter and has a deletion in the E1A and E1B region, impairing the ability of this virus to replicate and transform nonpermissive cells. The presence of FasL cDNA and absence of the E1 in this viral genome was confirmed by Southern blot analysis. The construction and propagation of ADV-FasL were performed in the FasL-resistant clone of 293 cells which was isolated by successive FasL transfections followed by limiting dilution. This 293 clone exhibited low expression of Fas by FACS analysis and relative resistance to FasL-stimulation in [^{51}Cr] assays or by inclusion of a Caspase inhibitor, N-benzyloxycarbonyl Val-Ala-Asp-flouromethylketone (2-VAD-fmk) in the cell culture media. The 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, L-glutamine and antibiotics. Cesium chloride-purified virus was dialyzed against PBS and diluted for storage in a 13% glycerol-PBS solution to yield a final concentration of $1x10^{12}$ viral particles/ml. All stocks were sterilized by passage through a 0.45 μm filter and evaluated for the presence of replication-competent virus. In the plaque assay, $0.9x10^{2}$ particles corresponded to 1 plaque forming unit on 293 cells.

Preparation of Neutrophils Enriched from Peripheral Blood

Fresh samples of venous blood from normal volunteers were collected into heparinized tubes. The human blood was mixed with an equal volume of 3% Dextran T-500 (Pharmacia) in 0.9% sodium chloride, and erythrocytes allowed to settle at room temperature for 20 - 30 min. The supernatant layer was centrifuged for 5 min at 350 x g, and the cell pellets were suspended in 0.9% sodium chloride. The cell suspension was underlain with cold Histopaque-1077 (Sigma) and centrifuged for 20 minutes at 1000 x g. For hypotonic lysis of contaminating erythrocytes, 10 ml of 0.2% sodium chloride was added to the cell pellets, the tube was gently vortexed for 20 s, and isotonicity was immediately restored with an equal volume of 1.6% sodium chloride. The preparation

contained greater than 97% PMN as seen on a Wright stained blood film. The mouse neutrophil-enriched PBL were obtained from Balb/c mice with modification, as described in Luo, Y. and Dorf, M.F. (1998) *Curr. Prot. Immun.* **3**:20. Briefly, the PBL were treated twice with hypotonic distilled water for 30 s each time. The preparation contained approximately 90% PMN as seen on a Wright stained blood film.

Animal experiments

Animal experiments were carried out in accordance with both institutional and NIH animal care regulations. Six-week-old female Balb/C, SCID, and SCID-*beige* (CB17-origin) mice were obtained from Charles River (Wilmington, MA) and Taconic (Germantown, NY), respectively, and kept in specific-pathogen-free environment. Cells were harvested with trypsin, incubated in growth medium at 37°C for one hour to recover surface molecules, washed three times with PBS, and resuspended in PBS. The CT26-neo and CT26-FasL cells (5×10^6) were inoculated subcutaneously in the flank.

Tumor size was followed in two perpendicular dimensions using calipers. For the adenoviral vector-mediated FasL gene transfer, 50µl of viral solution (1×10^{12} particle/ml) was injected into the tumor with a 26-gauge hypodermic needle after the tumor mass was established (~0.5 cm). One mouse of each group was sacrificed for histological examination of the tumor and major organs, and the remaining animals (Renca:n-6; CT26:n=4 each) were observed for tumor growth. For the inoculation of CT26-FasL and CT26-neo, one mouse of each group was sacrificed for examination on day 2 and the remaining mice (Balb/C:n=5 each; nude, SCID and SCID-beige:n=3 each) were followed. The histology of the major organs (liver, heart, lung, kidney, spleen, and thymus) was examined by microscopic observation of hematoxylin and eosin stained slides by an experienced pathologist.

CT26-CD95L cells or CT26-neo cells (10^5) were injected into right anterior chambers or subcutaneously into the flank of syngeneic Balb/c mice as described above. Tumor growth was monitored by observation and palpitation daily for 3 weeks. All tumor growth at the anterior chamber appeared before day

8 after injection and was confirmed by microscopic observation of hematoxy. The results are summarized below.

Table I

	Tumor Incidence	
Site of Injection	CT26-CD95L	CT26-Neo
Anterior chamber	5/5	4/4
Subcutaneous	0/5	4/4

Immunohistochemistry

Fresh-frozen tissues of CT26-FasL and CT26-neo tumor on day 2 were fixed with acetone. The section was first incubated with anti-Ly-6G (GR-1) monoclonal antibody (RB6-8C5) (Pharmingen) or an istotype control rat IgG. Biotinylated anti-rat IgG2b second antibody (Pharmingen) was added followed by the addition of preformed avidin biotinylated horseradish peroxidase complex. The signal was visualized by incubation in peroxidase substrate. The RB6-8C5 antibody reacts mainly with neutrophils and, to a lesser extent, with activated monocytes/macrophage as described in Julita, M.A. et al. (1988) *Eur. J. Immunol.* **18**:1819.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and example are intended to illustrate and not limit the scope of the invention. For example, any of the above-noted compositions and/or methods can be combined with known therapies or compositions. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

CLAIMS

What is claimed is:

1. A method for inhibiting a proinflammatory response in a suitable cell mixture, the method comprising administrating to the mixture an effective amount of an agent which suppresses a FasL proinflammatory response.

2. The method of claim 1, further comprising administering an effective amount of FasL.

3. The method of claim 1, wherein the cell mixture comprises neutrophil cells.

4. The method of claim 2, wherein FasL is administered by administration of a polynucleotide coding for FasL or soluble FasL.

5. The method of claim 1, wherein the immunosuppressive agent is administered by administration of a polynucleotide coding for the immunosuppressive agent.

6. The method of claim 4 or 5, wherein the administration comprises administration of the polynucleotide by a gene delivery vehicle.

7. The method of claim 6, wherein the gene delivery vehicle comprises a vehicle selected from the group of vehicles consisting of a liposome, a viral vector, and a plasmid.

8. The method of claim 2, wherein FasL is administered by administration of FasL protein or polypeptide.

9. The method of claim 2, wherein FasL is administered by administration of soluble FasL protein or polypeptide.

10. The method of claim 1, wherein the immunosuppressive agent is administered by the administration of an immunosuppressive protein or polypeptide.

11. The method of claim 1, wherein the agent is soluble Fas receptor protein or a polynucleotide encoding soluble Fas receptor protein.

12. The method of claim 2, wherein the agent stimulates Fas signalling and TGF-β signalling concurrently.

13. A method of inhibiting a FasL-mediated proinflammatory response in a subject, comprising administering to the subject an effective amount of an immunosuppressive agent that specifically inhibits the proinflammatory effect of FasL.

14. The method of claim 13, wherein the FasL-mediated proinflammatory response comprises neutrophil activation.

15. The method of claim 13, wherein the proinflammatory response comprises activation of the immune response mediated by cells of monocytic or granulocytic origin.

16. The method of claim 13, wherein the immunosuppressive agent is TGF-β1-5, an analog or biologically equivalent thereto.

17. The method of claim 16, wherein the TGF-β1-5 is administered by administration of a polynucleotide coding for the TGF-β1-5.

18. The method of claim 17, wherein the administration comprises administration of the polynucleotide by a gene delivery vehicle.

19. The method of claim 18, wherein the gene delivery vehicle comprises a vehicle selected from the group of vehicles consisting of a liposome, a viral vector, and a plasmid.

20. The method of claim 13, wherein the immunosuppressive agent is administered by administration of an immunosuppressive protein or polypeptide.

21. The method of claim 13, wherein immunosuppressive agent is TGF-β1-5, an analog or biologically equivalent thereto.

22. The method of claim 21, wherein the TGF-β1-5 is TGF-β1, an analog or biologically equivalent thereto.

23. A method for identifying agents which modulate FasL stimulation of a proinflammatory response, comprising:

(a) contacting a target cell mixture and a control cell mixture with FasL and an immunosuppressive agent;

(b) contacting the target cell mixture with a candidate therapeutic agent;

(c) assaying the target cell mixture for localized proinflammatory response; and

(d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates localized FasL stimulation of the proinflammatory response.

24. The method of claim 23, wherein the assaying of step (c) comprises assaying for modulation of a neutrophil mediated proinflammatory response.

25. The method of claim 23, wherein the assaying of step (c) comprises assaying for modulation of a proinflammatory response mediated by cells of monocytic or granulocytic origin.

26. A method for identifying agents which modulate FasL stimulation of a proinflammatory response, comprising:

(a) contacting a target cell mixture and a control cell mixture with FasL;

(b) contacting the target cell mixture with a candidate therapeutic agent and the control cell mixture with an immunosuppressive agent;

(c) assaying the target cell mixture for localized proinflammatory response; and

(d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates localized FasL stimulation of the proinflammatory response.

27. A method for identifying agents which modulate FasL stimulation of a proinflammatory response, comprising:

(a) contacting a target cell mixture and a control cell mixture with an effective amount of immunosuppressive agent;

(b) contacting the target cell mixture with an effective amount of candidate therapeutic agent and the control cell mixture with an effective amount of FasL:

(c) assaying the target cell mixture for localized proinflammatory response; and

(d) comparing the target cell mixture to the control cell mixture to determine if the agent modulates FasL-mediated stimulation of the proinflammatory response.

28. The method of any of claims 1, 23, 26 or 27, wherein the immunostimulatory agent comprises TGF-β1-5, an analog or variant thereof.

29. The method of any of claims 1, 23, 26 or 27, wherein the immunostimulatory agent comprises TGF-β1, an analog or mutant thereof.

30. A method for inhibiting the FasL-mediated proinflammatory response by administering an effective amount of agent which stimulates the production of TGF-β1-5.

31. The method of claim 30, wherein the agent is cyclosporin.

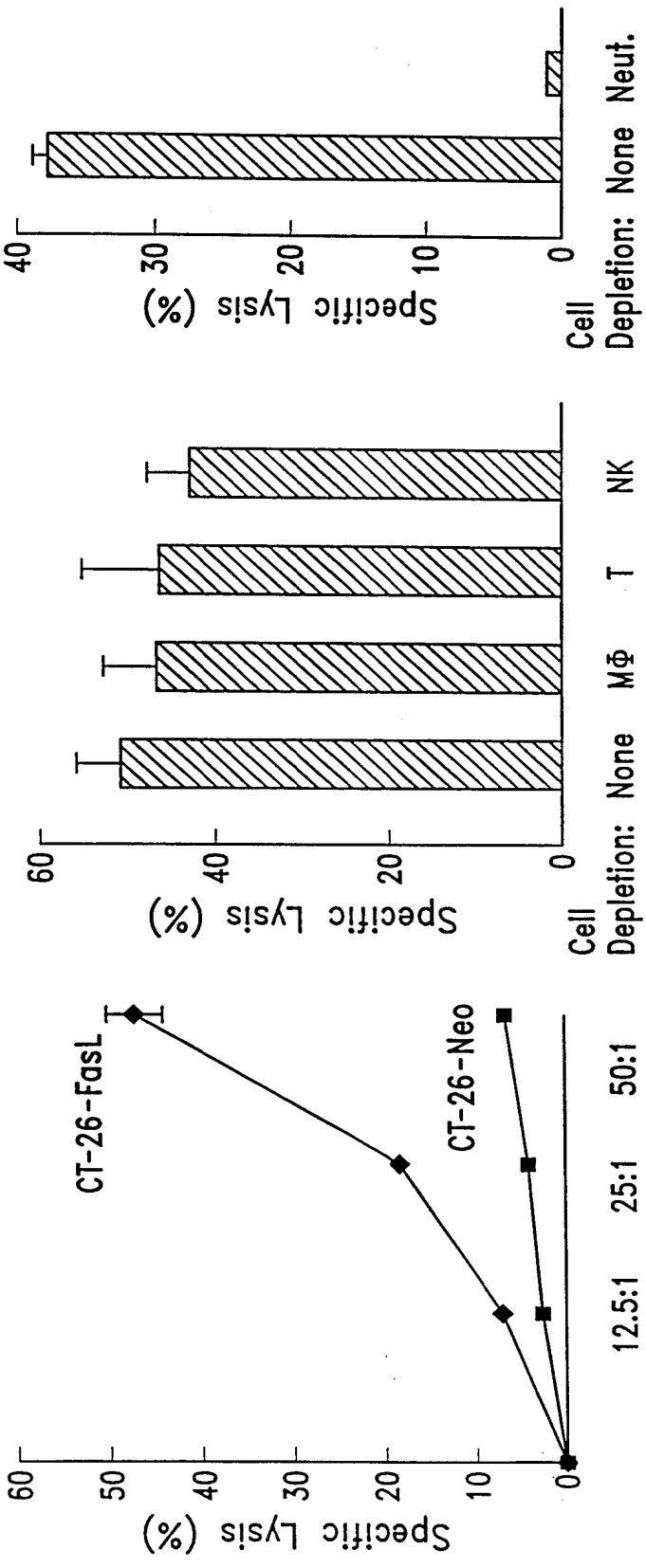

FIG. 1A

FIG. 1B

FIG. 1C

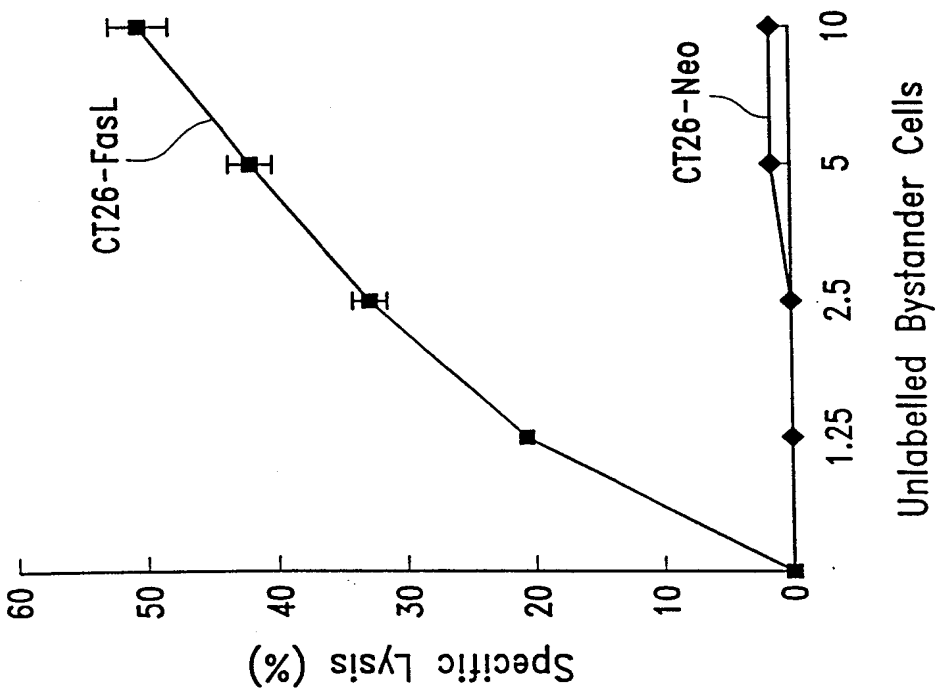
CT26-FasL
CT26-Neo
Specific Lysis (%)
0
10
20
30
40
50
60
1.25
2.5
5
10
Unlabelled Bystander Cells
(x10⁴)

FIG. 2B

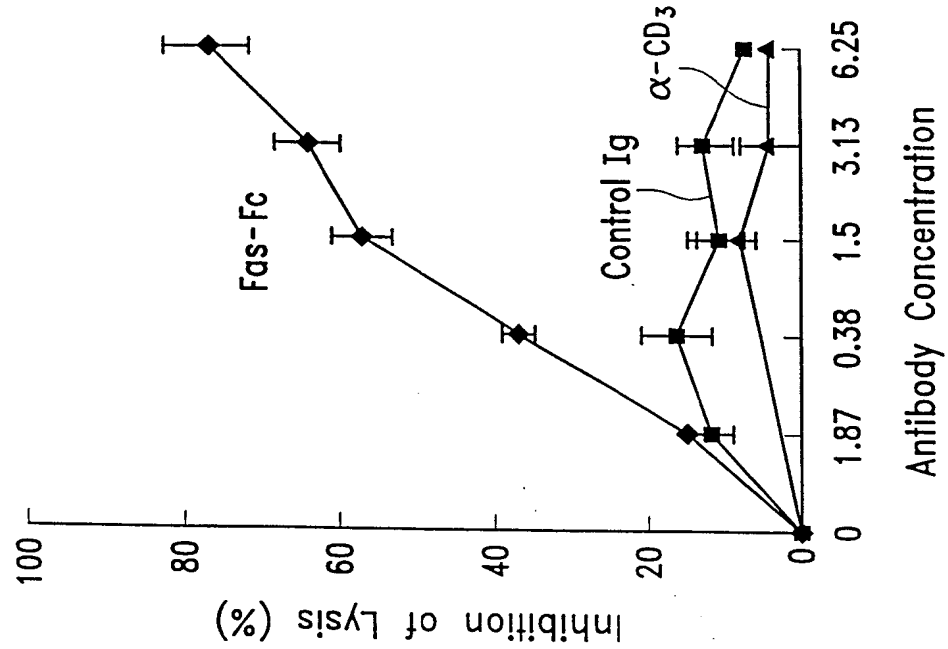
Fas-Fc
Control Ig
α-CD3
Inhibition of Lysis (%)
0
20
40
60
80
100
0
1.87
0.38
1.5
3.13
6.25
Antibody Concentration
(μg/ml)

FIG. 2A

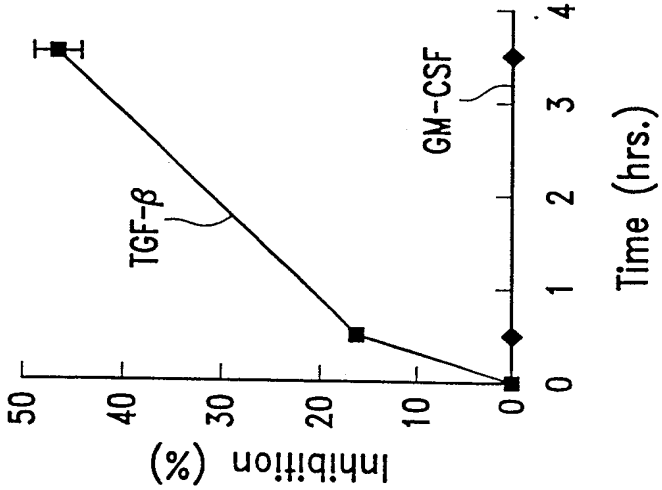

FIG. 3C

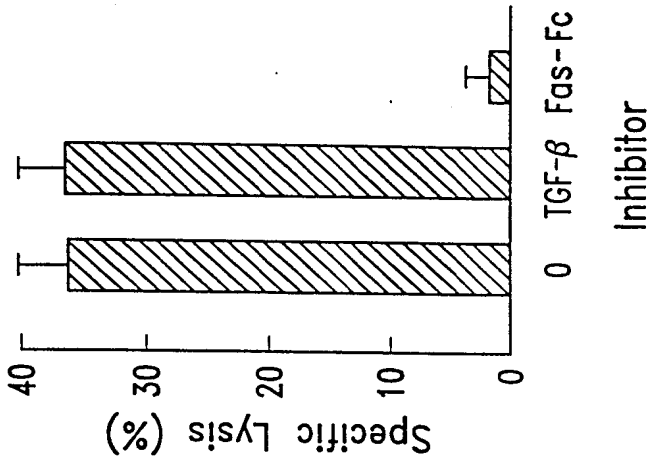

FIG. 3B

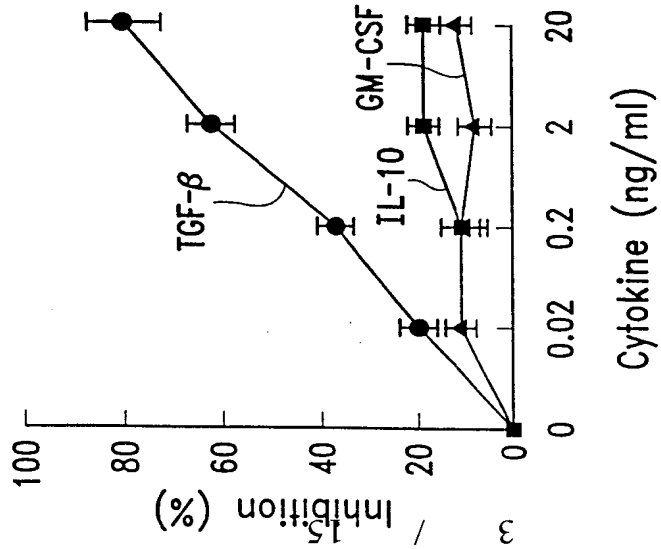

FIG. 3A

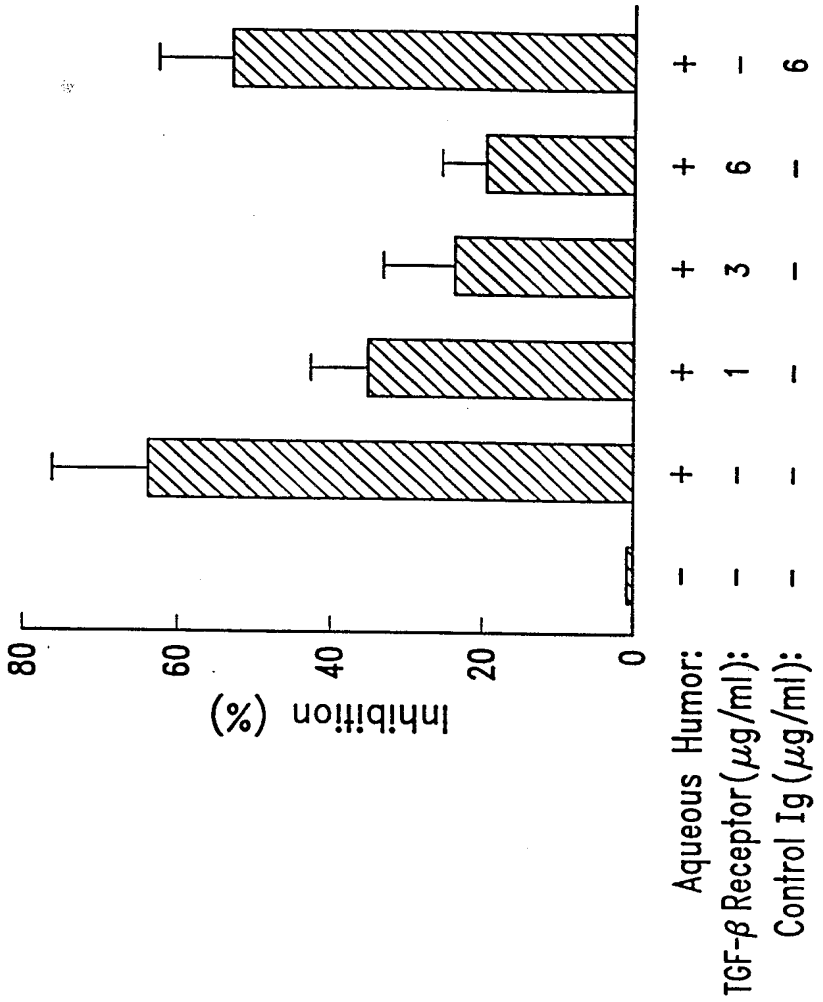
Inhibition (%)
0
20
40
60
80
Aqueous Humor: – + + + + + +
TGF-β Receptor (μg/ml): – – 1 3 6 –
Control Ig (μg/ml): – – – – – 6

FIG. 3E

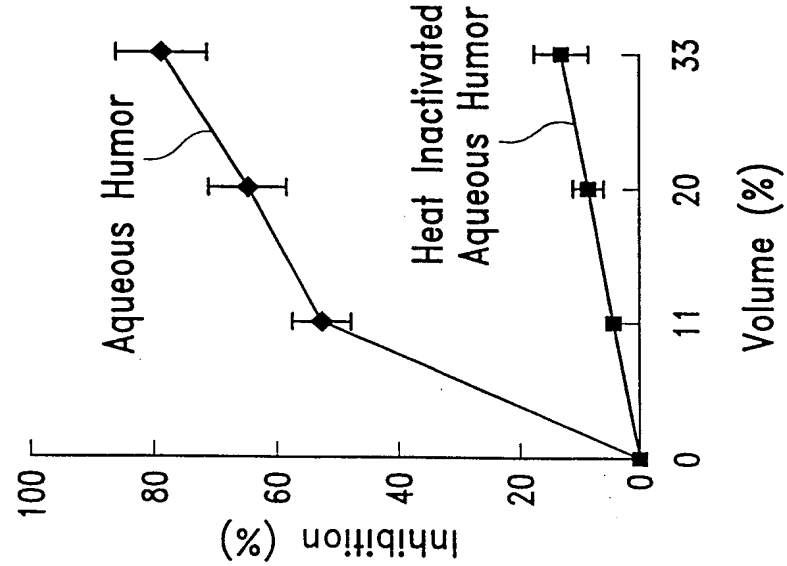
Inhibition (%)
0
20
40
60
80
100
Aqueous Humor
Heat Inactivated Aqueous Humor
0
11
20
33
Volume (%)

FIG. 3D

FIGURE 4A

```
                                                                                   50
                                   TCAGAGTCCTGTCCTTGACACTTCAGTCTCCACAAGACTGAGAGGAGGAAACCCTTTCCTGGGGCTGGGTGCC
                                        100
ATG CAG CAG CCC GTG AAT TAC CCA TGT CCC CAG ATC TAC TGG GTA GAC AGC AGT GCC ACT TCT CCT TGG GCT CCT
Met Gln Gln Pro Val Asn Tyr Pro Cys Pro Gln Ile Tyr Trp Val Asp Ser Ser Ala Thr Ser Pro Trp Ala Pro
                                         10                                      20
150                                                             200
CCA GGG TCA GTT TTT TCT TGT CCA TCC TCT GGG CCT AGA GGG CCA GGA CAA AGG AGA CCA CCG CCT CCA CCA CCA
Pro Gly Ser Val Phe Ser Cys Pro Ser Ser Gly Pro Arg Gly Pro Gly Gln Arg Arg Pro Pro Pro Pro Pro Pro
                     30                                      40                                  50
                                        250
CCT CCA TCA CCA CTA CCA CCG CCT TCC CAA CCA CCC CCG CTG CCT CCA CTA AGC CCT CTA AAG AAG AAG GAC AAC
Pro Pro Ser Pro Leu Pro Pro Pro Ser Gln Pro Pro Pro Leu Pro Pro Leu Ser Pro Leu Lys Lys Lys Asp Asn
                                         60                                      70
300                                                             350
ATA GAG CTG TGG CTA CCG GTG ATA TTT TTC ATG GTG CTG GTG GCT CTG GTT GGA ATG GGG TTA GGA ATG TAT CAA
Ile Glu Leu Trp Leu Pro Val Ile Phe Phe Met Val Leu Val Ala Leu Val Gly Met Gly Leu Gly Met Tyr Gln
                     80                                      90                                 100
                                        400
CTC TTT CAT CTA CAG AAG GAA CTG GCA GAA CTC CGT GAG TTC ACC AAC CAC AGC CTT AGA GTA TCA TCT TTT GAA
Leu Phe His Leu Gln Lys Glu Leu Ala Glu Leu Arg Glu Phe Thr Asn His Ser Leu Arg Val Ser Ser Phe Glu
                                        110                          *                  120
450                                                             500
AAG CAA ATA GCC AAC CCC AGC ACA CCC TCT GAA ACC AAA AAG CCA AGG AGT GTG GCC CAC TTA ACA GGG AAC CCC
Lys Gln Ile Ala Asn Pro Ser Thr Pro Ser Glu Thr Lys Lys Pro Arg Ser Val Ala His Leu Thr Gly Asn Pro
                 *                                          140                                 150
                                        550
CGC TCA AGG TCC ATC CCT CTG GAA TGG GAA GAC ACA TAT GGA ACT GCT TTG ATC TCT GGA GTG AAG TAT AAG AAA
Arg Ser Arg Ser Ile Pro Leu Glu Trp Glu Asp Thr Tyr Gly Thr Ala Leu Ile Ser Gly Val Lys Tyr Lys Lys
                                        160                                     170
600                                                             650
GGC GGC CTT GTG ATC AAT GAG GCT GGG TTG TAC TTC GTA TAT TCC AAA GTA TAC TTC CGG GGT CAG TCT TGC AAC
Gly Gly Leu Val Ile Asn Glu Ala Gly Leu Tyr Phe Val Tyr Ser Lys Val Tyr Phe Arg Gly Gln Ser Cys Asn
                    180                                     190                                 200
                                        700
AGC CAG CCC CTA AGC CAC AAG GTC TAT ATG AGG AAC TTT AAG TAT CCT GGG GAT CTG GTG CTA ATG GAG GAG AAG
Ser Gln Pro Leu Ser His Lys Val Tyr Met Arg Asn Phe Lys Tyr Pro Gly Asp Leu Val Leu Met Glu Glu Lys
                                        210                                     220
750                                                              900
AAG TTG AAT TAC TGC ACT ACT GGC CAG ATA TGG GCC CAC AGC AGC TAC CTA GGG GCA GTA TTT AAT CTT ACC GTT
Lys Leu Asn Tyr Cys Thr Thr Gly Gln Ile Trp Ala His Ser Ser Tyr Leu Gly Ala Val Phe Asn Leu Thr Val
                    230                                     240                     *           250
                                        850
GCT GAC CAT TTA TAT GTC AAC ATA TCT CAA CTC TCT CTG ATC AAT TTT GAG GAA TCT AAG ACC TTT TTT GGC TTA
Ala Asp His Leu Tyr Val Asn Ile Ser Gln Leu Ser Leu Ile Asn Phe Glu Glu Ser Lys Thr Phe Phe Gly Leu
                         *              260                                     270
900                                                         550
TAT AAG CTT TAA AGGAAAAAGCATTTTAGAATGATCTATTATTCTTTATCATGGATGCCAGGAATATTGTCTTCAATGAGAGTCTTCTTAAGACC
Tyr Lys Leu ***

  1000                                                    1050
AATTGAGCCACAAAGACCACAAGGTCCAACAGGTCAGCTACCCTTCATTTTCTAGAGGTCCATGGAGTGGTCCTTAATGCCTGCATCATGAGCCAGATG
   1100                                                    1150
GGAAGAAGACTGTTCCTGAGGAACATAAAGTTTTGGGCTGCTGTGTGCCAATGTAGAGGCAAAGAGAAGGAACTGTCTGATGTTAAATGGCCAAGAGCA
     1200                                                    1250
TTTTAGCCATTGAAGAAAAAAAAAACCTTTAAACTCACCTTCCAGGGTGGGTCTACTTGCTACCTCACAGGAGGCCGTCTTTTAGACACATGGTTGTGG
      1300                                                     1350
TATGACTATACAAGGGTGAGAAAGGATGCTAGGTTTCATGGATAAGCTAGAGACTGAAAAAAGCCAGTGTCCCATTGGCATCATCTTTATTTTTAACTG
       1400                                                     1450
ATGTTTTCTGAGCCCACCTTTGATGCTAACAGAGAAATAAGAGGGGTGTTTGAGGCACAAGTCATTCTCTACATAGCATGTGTACCTCCAGTGCAATGA
        1500                                                     1550
TGTCTGTGTGTGTTTTTATGTATGAGAGTAGAGCGATTCTAAAGAGTCACATGAGTACAACGCGTACATTACGGAGTACATATTAGAAACGTATGTGTT
         1600
ACATTTGATGCTAGAATATCTGAATGTTTCTTGCTA
```

Figure 4B-1

```
                                                                    GACGCTTCTGGGGAGTGAGGGAAGCGGTTTACGAGTGACTTGGCTGGAGCCTCAGGGGCGGGCACTGGCACGGA

ACACACCCTGAGGCCAGCCCTGGCTGCCCAGGCGGAGCTGCCTCTTCTCCCGCGGTTGGTGGACCCGCTCAGTACGGAGTTGGGGAA

GCTCTTTCACTTCGGAGGATTGCTCAACAACC

ATG CTG GGC ATC TGG ACC CTC CTA CCT CTG GTT CTT ACG TCT GTT GCT AGA TTA TCG TCC AAA AGT
Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala Arg Leu Ser Ser Lys Ser
                -10                                  -1  +1

GTT AAT GCC CAA GTG ACT GAC ATC AAC TCC AAG GGA TTG GAA TTG AGG AAG ACT GTT ACT ACA GTT
Val Asn Ala Gln Val Thr Asp Ile Asn Ser Lys Gly Leu Glu Leu Agr Lys Thr Val Thr Thr Val
      10                                  20

GAG ACT CAG AAC TTG GAA GGC CTG CAT CAT GAT GGC CAA TTC TGC CAT AAG CCC TGT CCT CCA GGT
Glu Thr Gln Asn Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro Pro Gly
  30                                  40

GAA AGG AAA GCT AGG GAC TGC ACA TGC AAT GGG GAT GAA CCA GAC TGC GTG CCC TGC CAA GAA GGG
Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro Asp Cys Val Pro Cys Gln Glu Gly
                                  60                                  70

AAG GAG TAC ACA GAC AAA GCC CAT TTT TCT TCC AAA TGC AGA AGA TGT AGA TTG TGT GAT GAA GGA
Lys Glu Tyr Thr Asp Lys Ala His Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Gly Gly
                                  80                                  90
```

Figure 4B-2

```
CAT GGC TTA GAA GTG GAA ATA AAC TGC ACC CGG ACC CAG AAT ACC AAG TGC AGA TGT AAA CCA AAC
His Gly Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg Cys Lys Pro Asn
                    100      *                                  110

TTT TTT TGT AAC TCT ACT GTA TGT GAA CAC TGT GAC CCT TGC ACC AAA TGT GAA CAT GGA ATC ATC
Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp Pro Cys Thr Lys Cys Glu His Gly Ile Ile
            120

AAG GAA TGC ACA CTC ACC AGC AAC ACC AAG TGC AAA GAG GAA GTG AAG AGA AAG GAA GTA CAG AAA
Lys Glu Cys Thr Leu Thr Ser Asn Thr Lys Cys Lys Glu Glu Val Lys Arg Lys Glu Val Gln Lys
    140                                 150                                 160

ACA TGC AGA AAG CAC AGA AAG GAA AAC CAA GGT TCT CAT GAA TCT CCA ACC TTA AAT CCT GAA ACA
Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr
                                170                                 180

GTG GCA ATA AAT TTA TCT GAT GTT GAC TTG AGT AAA TAT ATC ACC ACT ATT GCT GGA GTC ATG ACA
Val Ala Ile Asn Leu Ser Asp Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr
                            190                                 200

CTA AGT CAA GTT AAA GGC TTT GTT CGA AAG AAT GGT GTC AAT GAA GCC AAA ATA GAT GAG ATC AAG
Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys Ile Asp Glu Ile Lys
                    210                                 220
```

Figure 4B-3

```
AAT GAC AAT GTC CAA GAC ACA GCA GAA CAG AAA GTT CAA CTG CTT CGT AAT TGG CAT CAA CTT CAT
Asn Asp Asn Val Gln Asp Thr Ala Glu Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His
           230                                 240

GGA AAG AAA GAA GCG TAT GAC ACA TTG ATT AAA GAT CTC AAA AAA GCC AAT CTT TGT ACT CTT GCA
Gly Lys Lys Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala
    250                                 260                                 270

GAG AAA ATT CAG ACT ATC ATC CTC AAG GAC ATT ACT AGT GAC TCA GAA AAT TCA AAC TTC AGA AAT
Glu Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn Phe Arg Asn
                                280                                 290

GAA ATC CAA AGC TTG GTC TAG AGTGAAAAACAACAAATTCAGTTCTGAGTATATGCAATTAGTGTTTGAAAAGATTCTTA
Glu Ile Gln Ser Leu Val End
                    298

ATAGCTGGCTGTAAATACTGCTTGGTTTTTTACTGGGTACATTTTATCATTTATTAGCGCTGAAGAGCCAACATATTTGTAGATTTTT

AATATCTCATGATTCTGCCTCCAAGGATGTTTAAAATCTAGTTGGGAAAACAAACTTCATCAAGAGTAAATGCAGTGGCATGCTAAGT

ACCCAAATAGGAGTGTATGCAGAGGATGAAAGATTAAGATTATGCTCTGGCATCTAACATATGATTCTGTAGTATGAATGTAATCAGT

GTATGTTAGTACAAATGTCTATCCACAGGCTAACCCCACTCTATGAATCAATAGAAGAAGCTATGACCTTTTGCTGAAATATCAGTTA

CTGAACAGGCAGGCCACTTTGCCTCTAAATTACCTCTGATAATTCTAGAGATTTTACCATATTTCTAAACTTTGTTTATAACTCTGAG
```

Figure 4B-4

AAGATCATATTTATGTAAAGTATATGTATTTGAGTGCAGAATTTAAATAAGGCTCTACCTCAAAGACCTTTGCACAGTTTATTGGTGT

CATATTATACAATATTTCAATTGTGAATTCACATAGAAAACATTAAATTATAATGTTTGACTATTATATATGTGTATGCATTTTACTG

GCTCAAAACTACCTACTTCTTTCTCAGGCATCAAAAGCATTTTGAGCAGGAGAGTATTACTAGAGCTTTGCCACCTCTCCATTTTTGC

CTTGGTGCTCATCTTAATGGCCTAATGCACCCCCAAACATGGAAATATCACCAAAAAATACTTAATAGTCCACCAAAAGGCAAGACTG

CCCTTAGAAATTCTAGCCTGGTTTGGAGATACTAACTGCTCTCAGAGAAAGTAGCTTTGTGACATGTCATGAACCCATGTTTGCAATC

AAAGATGATAAAATAGATTCTTATTTTTCCCCCACCCCCGAAAATGTTCAATAATGTCCCATGTAAAACCTGCTACAAATGGCAGCTT

ATACATAGCAATGGTAAAATCATCATCTGGATTTAGGAATTGCTCTTGTCATACCCTCAAGTTTCTAAGATTTAAGATTCTCCTTACT

ACTATCCTACGTTTAAATATCTTTGAAAGTTTGTATTAAATGTGAATTTTAAGAAATAATATTTATATTTCTGTAAATGTAAACTGTG

AAGATAGTTATAAACTGAAGCAGATACCTGGAACCACCTAAAGAACTTCCATTTATGGAGGATTTTTTTGCCCCTTGTGTTTGGAATT

ATAAAATATAGGTAAAAGTACGTAATTAAATAATGTTTTTG

Figure 5-1

```
GAATTCCGGG TCCCGTCCTT GACACCTCAG CCTCTACAGG ACTGAGAAGA AGTAAAACCG      60

  TTTGCTGGGG CTGGCCTGAC TCACCAGCTG CC ATG CAG CAG CCC TTC AAT TAC      113
                                      Met Gln Gln Pro Phe Asn Tyr
                                        1               5

  CCA TAT CCC CAG ATC TAC TGG GTG GAC AGC AGT GCC AGC TCT CCC TGG      161
  Pro Tyr Pro Gln Ile Tyr Trp Val Asp Ser Ser Ala Ser Ser Pro Trp
           10                  15                  20

  GCC CCT CCA GGC ACA GTT CTT CCC TGT CCA ACC TCT GTG CCC AGA AGG      209
  Ala Pro Pro Gly Thr Val Leu Pro Cys Pro Thr Ser Val Pro Arg Arg
       25                  30                  35

  CCT GGT CAA AGG AGG CCA CCA CCA CCA CCG CCA CCG CCA CCA CTA CCA      257
  Pro Gly Gln Arg Arg Pro Pro Pro Pro Pro Pro Pro Pro Pro Leu Pro
   40                  45                  50                  55

  CCT CCG CCG CCG CCG CCA CCA CTG CCT CCA CTA CCG CTG CCA CCC CTG      305
  Pro Pro Pro Pro Pro Pro Pro Leu Pro Pro Leu Pro Leu Pro Pro Leu
                  60                  65                  70

  AAG AAG AGA GGG AAC CAC AGC ACA GGC CTG TGT CTC CTT GTG ATG TTT      353
  Lys Lys Arg Gly Asn His Ser Thr Gly Leu Cys Leu Leu Val Met Phe
                  75                  80                  85

  TTC ATG GTT CTG GTT GCC TTG GTA GGA TTG GGC CTG GGG ATG TTT CAG      401
  Phe Met Val Leu Val Ala Leu Val Gly Leu Gly Leu Gly Met Phe Gln
           90                  95                  100

  CTC TTC CAC CTA CAG AAG GAG CTG GCA GAA CTC CGA GAG TCT ACC AGC      449
  Leu Phe His Leu Gln Lys Glu Leu Ala Glu Leu Arg Glu Ser Thr Ser
       105                 110                 115

  CAG ATG CAC ACA GCA TCA TCT TTG GAG AAG CAA ATA GGC CAC CCC AGT      497
  Gln Met His Thr Ala Ser Ser Leu Glu Lys Gln Ile Gly His Pro Ser
  120                 125                 130                 135

  CCA CCC CCT GAA AAA AAG GAG CTG AGG AAA GTG GCC CAT TTA ACA GGC      545
  Pro Pro Pro Glu Lys Lys Glu Leu Arg Lys Val Ala His Leu Thr Gly
                  140                 145                 150

  AAG TCC AAC TCA AGG TCC ATG CCT CTG GAA TGG GAA GAC ACC TAT GGA      593
  Lys Ser Asn Ser Arg Ser Met Pro Leu Glu Trp Glu Asp Thr Tyr Gly
             155                 160                 165

  ATT GTC CTG CTT TCT GGA GTG AAG TAT AAG AAG GGT GGC CTT GTG ATC      641
  Ile Val Leu Leu Ser Gly Val Lys Tyr Lys Lys Gly Gly Leu Val Ile
         170                 175                 180

  AAT GAA ACT GGG CTG TAC TTT GTA TAT TCC AAA GTA TAC TTC CGG GGT      689
  Asn Glu Thr Gly Leu Tyr Phe Val Tyr Ser Lys Val Tyr Phe Arg Gly
      185                 190                 195

  CAA TCT TGC AAC AAC CTG CCC CTG AGC CAC AAG GTC TAC ATG AGG AAC      737
  Gln Ser Cys Asn Asn Leu Pro Leu Ser His Lys Val Tyr Met Arg Asn
  200                 205                 210                 215

  TCT AAG TAT CCC CAG GAT CTG GTG ATG ATG GAG GGG AAG ATG ATG AGC      785
  Ser Lys Tyr Pro Gln Asp Leu Val Met Met Glu Gly Lys Met Met Ser
                  220                 225                 230
```

Figure 5-2

```
TAC TGC ACT ACT GGG CAG ATG TGG GCC CGC AGC AGC TAC CTG GGG GCA    833
Tyr Cys Thr Thr Gly Gln Met Trp Ala Arg Ser Ser Tyr Leu Gly Ala
            235                 240                 245

GTG TTC AAT CTT ACC AGT GCT GAT CAT TTA TAT GTC AAC GTA TCT GAG    881
Val Phe Asn Leu Thr Ser Ala Asp His Leu Tyr Val Asn Val Ser Glu
        250                 255                 260

CTC TCT CTG GTC AAT TTT GAG GAA TCT CAG ACG TTT TTC GGC TTA TAT    929
Leu Ser Leu Val Asn Phe Glu Glu Ser Gln Thr Phe Phe Gly Leu Tyr
    265                 270                 275

AAG CTC TAAGAGAAGC ACTTTGGGAT TCTTTCCATT ATGATTCTTT GTTACAGGCA     985
Lys Leu
280

CCGAGAATGT TGTATTCAGT GAGGGTCTTC TTACATGCAT TTGAGGTCAA GTAAGAAGAC 1045

ATGAACCAAG TGGACCTTGA GACCACAGGG TTCAAAATGT CTGTAGCTCC TCAACTCACC 1105

TAATGTTTAT GAGCCAGACA AATGGAGGAA TATGACGGAA GAACATAGAA CTCTGGGCTG 1165

CCATGTGAAG AGGGAGAAGC ATGAAAAAGC AGCTACCAGG TGTTCTACAC TCATCTTAGT 1225

GCCTGAGAGT ATTTAGGCAG ATTGAAAAGG ACACCTTTTA ACTCACCTCT CAAGGTGGGC 1285

CTTGCTACCT CAAGGGGGAC TGTCTTTCAG ATACATGGTT GTGACCTGAG GATTTAAGGG 1345

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CCCACTGGCA GCATCTTCAC TTCTAAATGC ATATCCTGAG CCATCGGTGA AACTAACAGA 1465

TAAGCAAGAG AGATGTTTTG GGGACTCATT TCATTCCTAA CACAGCATGT GTATTTCCAG 1525

TGCAATTGTA GGGGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTATGAC TAAAGAGAGA 1585

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ATGCTTCCTG ACAATCAACT CTAATAGTGC TTAAAAATCA TTGATTGTCA GCTACTAATG 1705

ATGTTTTCCT ATAATATAAT AAATATTTAT GTAGATGTGC ATTTTTGTGA AATGAAAACA 1765

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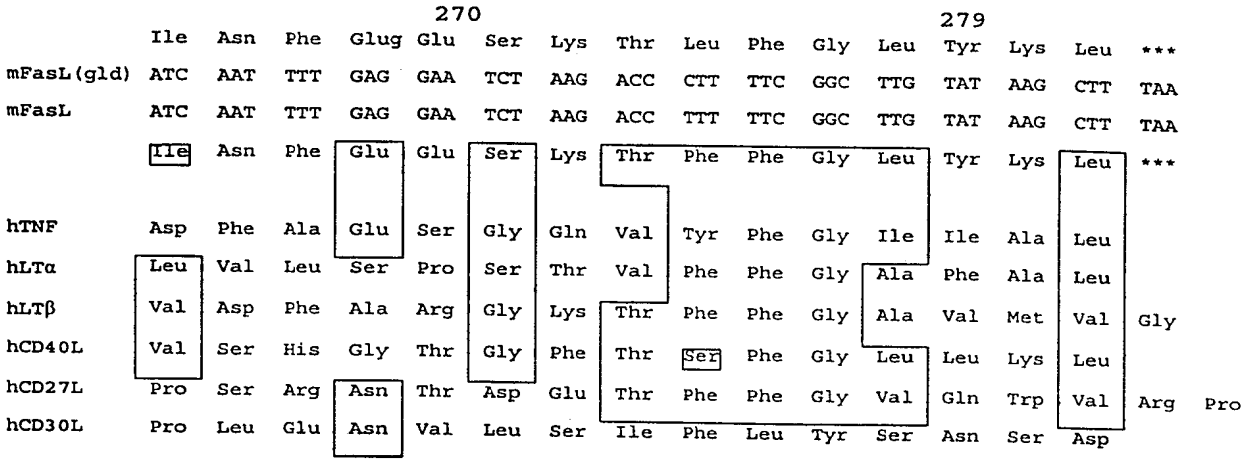
FIGURE 6


270
279
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mFasL ATC AAT TTT GAG GAA TCT AAG ACC TTT TTC GGC TTG TAT AAG CTT TAA
Ile Asn Phe Glu Glu Ser Lys Thr Phe Phe Gly Leu Tyr Lys Leu ***
hTNF Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
hLTα Leu Val Leu Ser Pro Ser Thr Val Phe Phe Gly Ala Phe Ala Leu
hLTβ Val Asp Phe Ala Arg Gly Lys Thr Phe Phe Gly Ala Val Met Val Gly
hCD40L Val Ser His Gly Thr Gly Phe Thr Ser Phe Gly Leu Leu Lys Leu
hCD27L Pro Ser Arg Asn Thr Asp Glu Thr Phe Phe Gly Val Gln Trp Val Arg Pro
hCD30L Pro Leu Glu Asn Val Leu Ser Ile Phe Leu Tyr Ser Asn Ser Asp

FIGURE 7

```
   1  gtgaccgagc ggcgcggacg gccgcctgcc ccctctgcca cctggggcgg tgcgggcccg
  61  gagcccggag cccgggtagc gcgtagagcc ggcgcgatgc acgtgcgctc actgcgagct
 121  gcggcgccgc acagcttcgt ggcgctctgg gcacccctgt tcctgctgcg ctccgccctg
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 421  gccgtcttca gtacccaggg ccccctctg gccagcctgc aagatagcca tttcctcacc
 481  gacgccgaca tggtcatgag cttcgtcaac ctcgtggaac atgacaagga attcttccac
 541  ccacgctacc accatcgaga gttccggttt gatctttcca agatcccaga aggggaagct
 601  gtcacggcag ccgaattccg gatctacaag gactacatcc gggaacgctt cgacaatgag
 661  acgttccgga tcagcgttta tcaggtgctc caggagcact tgggcaggga atcggatctc
 721  ttcctgctcg acagccgtac cctctgggcc tcggaggagg gctggctggt gtttgacatc
 781  acagccacca gcaaccactg ggtggtcaat ccgcggcaca acctgggcct gcagctctcg
 841  gtggagacgc tggatgggca gagcatcaac cccaagttgg cgggcctgat tgggcggcac
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1021  aaccaggaag ccctgcggat ggccaacgtg gcagagaaca gcagcagcga ccagaggcag
1081  gcctgtaaga agcacgagct gtatgtcagc ttccgagacc tgggctggca ggactggatc
1141  atcgcgcctg aaggctacgc cgcctactac tgtgaggggg agtgtgcctt ccctctgaac
1201  tcctacatga acgccaccaa ccacgccatc gtgcagacgc tggtccactt catcaacccg
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1441  cattgctc
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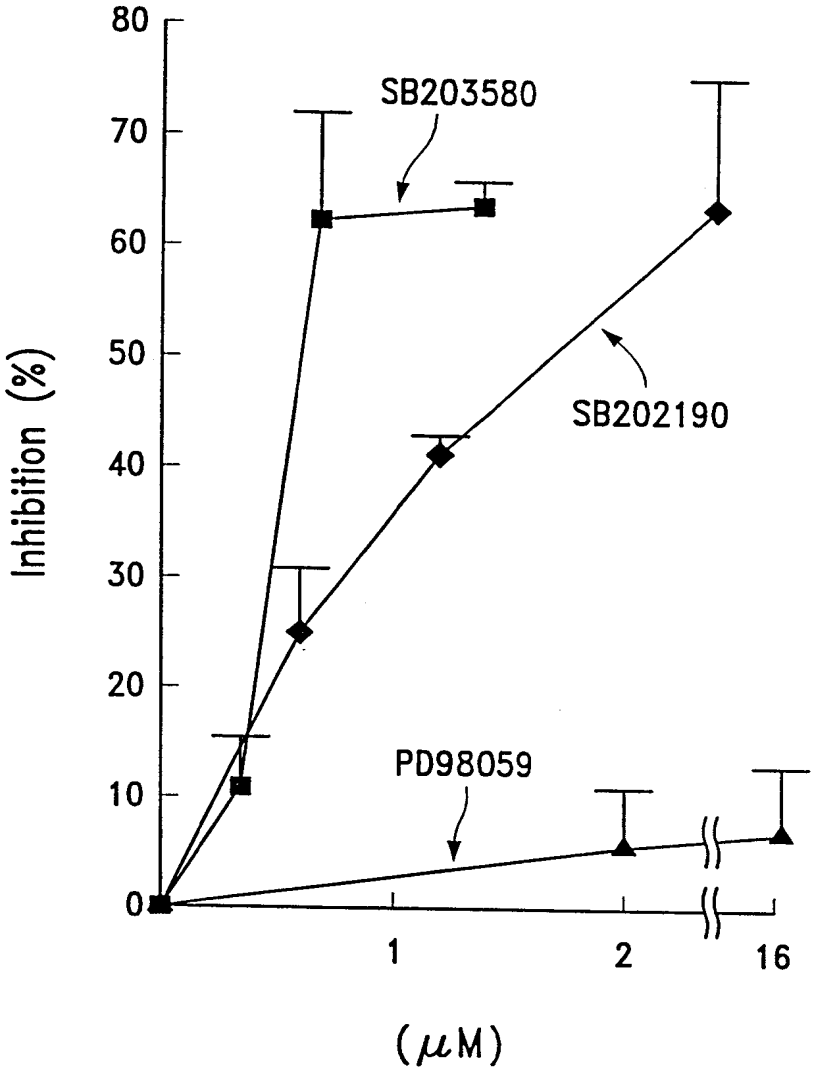
80
70
60
50
40
30
20
10
0
Inhibition (%)
SB203580
SB202190
PD98059
1
2
16
(μM)

FIG. 8A

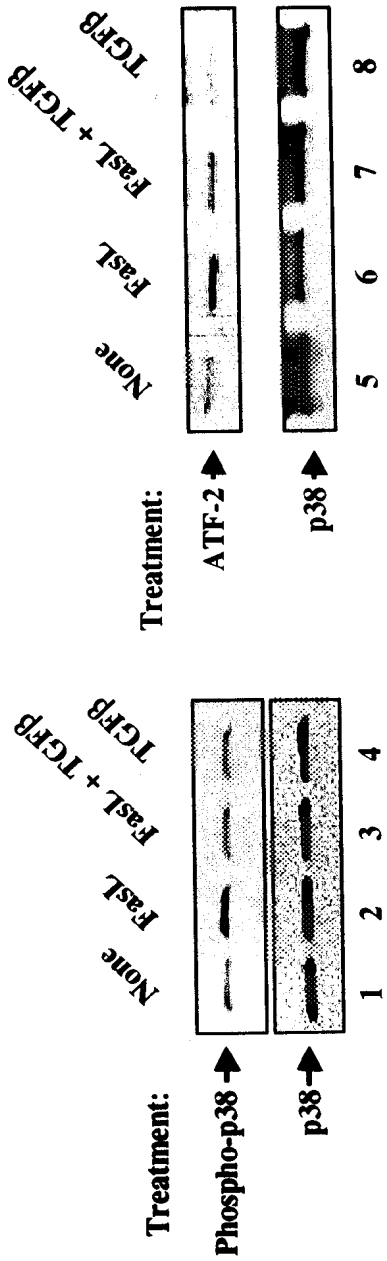
Treatment:
None
FasL
FasL + TGFβ
TGFβ
ATF-2
p38
5
6
7
8
Phospho-p38
p38
1
2
3
4

Figure 8B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/14771

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 A61K38/17 A61K38/18 A61K48/00 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEINO ET AL: "ANTITUMOR EFFECT OF LOCALLY PRODUCED CD95 LIGAND" NATURE MEDICINE, vol. 3, no. 2, February 1997, pages 165-170, XP002084129 cited in the application see page 165 see abstract see page 169, paragraph 3 --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1998	14/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/14771

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	KANG ET AL: "FAS LIGAND EXPRESSION IN ISLETS OF LANGERHANS DOES NOT CONFER IMMUNE PRIVILEGE AND INSTEAD TARGETS THEM FOR RAPID DESTRUCTION" NATURE MEDICINE, vol. 3, no. 7, July 1997, pages 738-743, XP002086213 cited in the application see page 738 see abstract see page 741 - page 742 see 'Discussion' ---	1-31
Y	ALLISON ET AL: "TRANSGENIC EXPRESSION OF CD95 LIGAND ON ISLET BETA CELLS INDUCES A GRANULOCYTIC INFILTRATION BUT DOES NOT CONFER IMMUNE PRIVILEGE UPON ISLET ALLOGRAFTS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 94, April 1997, pages 3943-3947, XP002086214 see page 3943 see abstract ---	1-31
Y	SHIHAB ET AL: "CYCLOSPORINE NEPHROPATHY: PATHOPHYSIOLOGY AND CLINICAL IMPACT" SEMINARS IN NEPHROLOGY, vol. 16, no. 6, 1996, pages 536-547, XP002086215 cited in the application see page 536 see abstract ---	1-10, 13-15, 20, 23-27, 30,31
Y	WO 90 00900 A (AMGEN INC) 8 February 1990 see page 3, line 14 - line 22 ---	1-10, 12-29
Y	WO 94 06476 A (IMMUNEX CORP) 31 March 1994 see page 16, line 27 - page 17, line 20 ---	1-11, 13-15, 20,23-27
P,Y	KANG S-M ET AL: "Rapid neutrophilic destruction of Fas ligand expressing cell lines in vivo reveals a novel proinflammatory function of Fas ligand." SURGICAL FORUM 48 (0). 1997. 451-453. ISSN: 0071-8041, XP002084132 see the whole document -----	1-31

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 14771

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-12, 28-31 partially and 13-22 completely are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/14771

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9000900 A	08-02-1990	AU 4056089 A	19-02-1990
WO 9406476 A	31-03-1994	AU 670125 B	04-07-1996
		AU 4920993 A	12-04-1994
		CA 2123593 A	31-03-1994
		EP 0620739 A	26-10-1994
		JP 7504203 T	11-05-1995
		NO 941780 A	15-07-1994
		NZ 256293 A	24-06-1997
		US 5605690 A	25-02-1997